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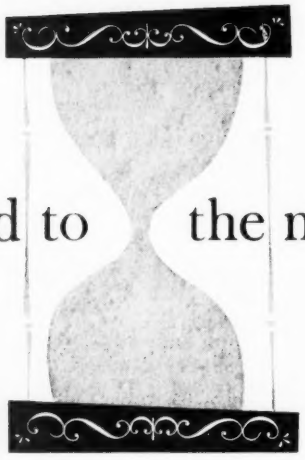

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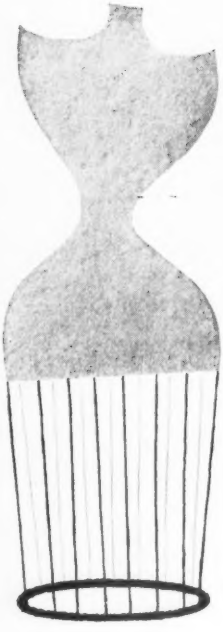

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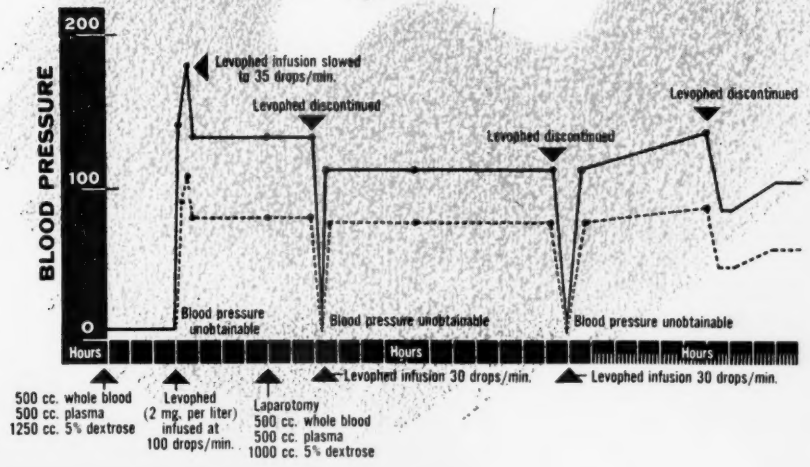

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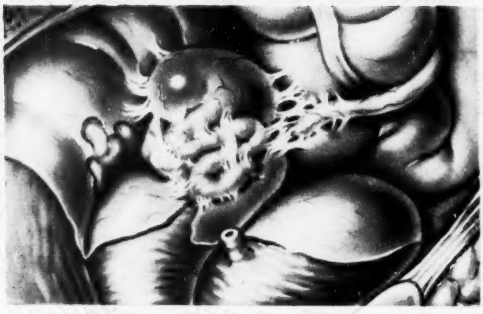

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1. Greene, C. C.: Kentucky M. J. *50*:8, 1952.
2. Stevenson, C. S., et al.: Am. J. Obst. & Gynec. *61*:498, 1951.

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PERMANENT IMPLANTATION OF MULTILEAD ELECTRODES IN THE BRAIN*

JOSE M.R. DELGADO†

Anesthesia and restraint of the animal subject are the two main factors which may modify responses evoked by stimulation of the brain. However, much of the information concerning brain physiology is of necessity based upon experiments performed under one or both of these limiting circumstances. The techniques developed thus far for the study of brain function in unanesthetized animals are described in the following paragraphs.

1. Hess,[7] one of the pioneers in the field, was the first successfully to implant electrodes in the diencephalic structures of the cat. Using the sutures of the skull as reference points, a metallic frame with a superstructure is screwed to the parietal bone. This superstructure serves to support the electrodes while they are being driven into the brain. It is then removed. Remaining are the metal frame and the electrodes. Since Hess's experiments were of short duration, aseptic precautions were not taken.

2. Variations of the remote control technique have been used by Chaffee and Light,[1] Clark and Ward,[3] Fender,[5] Loucks,[12] and Mauro.[14] In this method a small induction coil having terminals inside the brain is placed under the skin. Stimulation is accomplished by applying a magnetic field to the head of the animal and then inducing a current in the subcutaneous coil. The main advantage of this is that no leads pierce the skin. Some of the difficulties and limitations of the remote control techniques are: (a) the number of electrodes is limited to one or two; (b) it is impossible to record electrical activity from the brain; and (c) it is difficult to control the parameters of stimulation accurately.

3. Pachon and Delmas,[15] Hunter and Jasper,[9] Clark and Ward,[2, 4] Hoagland,[8] Lubinska and Konorski,[13] Rheinberger and Jasper,[16] and Gastaut[6] used variations of a general procedure that consisted essentially of screwing to the skull a small plastic or stainless steel base in which were inserted the electrodes (accomplished either with or without a Horsley-Clarke apparatus). In some instances aseptic procedure was followed.

The present paper describes a technique for aseptically implanting a large number of electrodes (fourteen to forty). This makes it possible to study individually or simultaneously many points on the surface and in the

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interior of the brain for periods of as long as a year without any resulting ill effects to the animal subject. During the experiments the animal has complete freedom of movement.

Method

The two types of electrodes which have been employed are: (i) a "needle electrode" for study of regions in the interior of the brain; and (ii) a "plate electrode" for study of areas on the brain surface. There are slight differences in the electrodes depending on the species of animal in which they are to be used. Following are descriptions of the kinds adapted for use on the cat or monkey.

Needle electrode (Fig. 1). Enameled stainless steel wire 0.005 inch in diameter is used. Pieces of approximately 18 cm. are straightened by stretching, and the tips which are to form the needle are scraped bare of enamel for 1 mm. The opposite ends are also scraped for 15 mm., and then the perfect isolation of each electrode is tested by using an ohmmeter.

After the test one wire is held between the left thumb and forefinger and another wire is placed beside it so that the tips are 2 mm. apart (the distance may change in accordance with the needs of each experiment). By means of a small glass rod the wires are cemented with liquid plexiglas. Several coats are applied. When these are dry, a third wire is cemented in place in the same way as before; then in succession four more wires are cemented to form a single body of seven wires with each tip 2 mm. apart. These seven wires form an electrode needle that is 0.5 mm. in diameter. The opposite tips of the electrodes are soldered in some identifiable order to a miniature seven-pin socket.

The group of seven electrodes is protected by polyethylene tubing, which in the portion close to the socket is exactly adapted to the electrodes by means of a small flame and in the distal part is occluded and attached to the electrodes with liquid plexiglas.

Each wire is again tested with the ohmmeter (one pole in the socket and the other in the free tip of the wire). After washing it with soap and water, the electrode needle is immersed in Zephiran 1/1.000 for sterilization.

Plate electrode (Fig. 1). A piece of polyethylene tubing approximately 15 cm. long and 1.5 mm. in diameter is slit open at one end for 30 mm. This part is spread out and pressed with a warm iron (not too warm) to form a small plate at the end of the tubing. Using a sewing needle, seven holes are punched 2 or 3 mm. apart along the midline of this plate.

Seven pieces of enameled stainless steel wire (0.005) are cut and straightened by stretching, and one tip of each is melted to form a small ball (see Riley).[17] After assuring the isolation of all the electrodes with the ohmmeter, they are put through the individual holes prepared for them in the polyethylene plate; then all are threaded through the tubing.

Each electrode is soldered in some specific order to the pins of a miniature radio tube socket, and with liquid plexiglas the electrodes are fixed to the posterior part of the plate. Employing suction, a small amount of liquid plexiglas is passed through the tubing and dried in place with a current of air. The tubing is closed by applying a flame to the end close to the socket and liquid plexiglas to the distal extremity.

The electrode may be used with the small balls protruding from the plastic plate, but it is better to abrade the balls with a fine emery wheel to form minute flat buttons.

Surgery for Implantation

Needle electrodes. The animal is operated upon under general anesthesia (e.g. Nembutal), and aseptic precautions are taken which include the sterilization of the stereotaxic instrument. When the head and the posterior part of the neck have been shaved, the animal is placed in the Horsley-Clarke apparatus.

An incision, 4 or 5 cm. long, is made exactly in the midline of the head ending at the occipital protuberance. Another very small incision is made in the posterior part of the neck through which a rubber tubing is passed beneath the skin in the direction of the principal incision. By means of a strong needle the base of the occipital protuberance is perforated and strong sutures are then passed through.

The coordinates of the stereotaxic instrument are adjusted so that the exact point may be marked on the skull. Using a trephine 1.5 mm. in diameter this point is opened. The previously placed rubber tubing is used to pass the needle electrode beneath the skin of the neck so that it may be attached to the micromanipulator of the Horsley-Clarke instrument. The dura is punctured and the electrodes are inserted to the desired depth. The small hole in the skull is closed with dental cement, which holds the electrodes firmly in position. Only a minute quantity should be used in order to avoid the formation of an excessive protuberance on the skull.

When the cement is dry (in about five minutes), the needle electrode is removed from the micromanipulator and is curved 90 degrees for application to the surface of the skull. Then the electrodes are fixed to the occipital protuberance by means of the sutures that were placed earlier (Fig. 2).

The implantation is repeated for each group of electrodes. The muscle, subcutaneous tissue, and skin (intradermic) are sutured, and the operation is finished.

Plate electrodes. The surgical technique for the implantation of plate electrodes in order to study the surface of the brain is similar to that described above with the following differences. The Horsley-Clarke instrument is not needed. The trephining of the skull must be about 1 cm. in diameter. The dura is opened with a fine knife and the plate electrode is slipped between the brain and the dura to the desired position. (It is also possible to make the craniotomy larger and to place the electrodes under visual control.) The electrodes are fixed in position by means of sutures passed through the bone at the edge of the orifice of trephining. The electrodes are rigid enough to stay placed, supported by the sutures, and at the same time flexible enough to remain in position in spite of cerebral pulsatile movements.

Adaptation of the technique to different circumstances. In a small animal such as the mouse there is no occipital protuberance for fixing the electrodes and it is necessary to perforate the occipital bone without piercing

the dura in order to pass the sutures through. In this animal it is necessary to reduce the size of the burr hole, thus a trephine of only 0.5 mm. is used. Also, the distance between the electrodes which make up the needle or the plate must be reduced to 1 mm. (instead of 2 mm.), but of course this distance depends upon the type of experiment.

If one wishes to study only a few points in several different regions of the brain (for example, eight zones situated at different coordinates), needles or plates with only two, three, or four electrodes may be used. In such a case it is possible to solder the electrodes to the miniature radio tube socket after the implantation. During the soldering, the electrodes must be cooled with ice in order to avoid conduction of heat to the brain. If the needle electrode is made up of only three fine wires, it is convenient to increase the size of the first one to give rigidity to the needle. Therefore, the first electrode will have a diameter of .010 inches instead of .005 inches.

The sockets of the electrodes must be protected by a plastic collar for support and to avoid interference with the movements of the animal (Fig. 3). Cats and dogs accept the collar without difficulty, but mice and monkeys try to remove it during the first days. For this reason either of these species must become accustomed to a collar for two weeks before the operation. In experiments of long duration the normal head motility of an animal may cause breakage of the electrodes because of the continuous bending at the point of suturing to the occipital protuberance. To avoid this, electrodes must be protected by a spring made by winding a fine stainless steel wire around the plastic tubing. The spiral should start 2 to 3 mm. before the suture and end 10 mm. behind it. This flexible spiral buffers the movements and, furthermore, may be used as an indifferent electrode for monopolar stimulation or recording.

Discussion

The implanted electrodes are well tolerated. No infection occurs despite the omission of antibiotics, and the animals remain in such excellent health that they jump and play with their companions without displaying any ill effects whatsoever.

Each needle of seven electrodes cemented and protected by plexiglas has a diameter of 0.5 mm. The remaining length of the electrode, which is protected with polyethylene tubing, has a diameter of 1.5 mm. This small size, aseptic technique, and the use of materials that cause minimum tissue reaction[10, 11] allow long-term placement of the electrodes within the brain. Cerebral damage is limited to that resulting from the insertion of the needle which causes a small gliosis (Fig. 4), and the electrodes may stay in for several months or even a year without detriment to the animal. In the case of plate electrodes there is no damage at all.

The stability of the position of the electrodes—both surface and depth—makes it possible to carry on an investigation for as long a time as is neces-

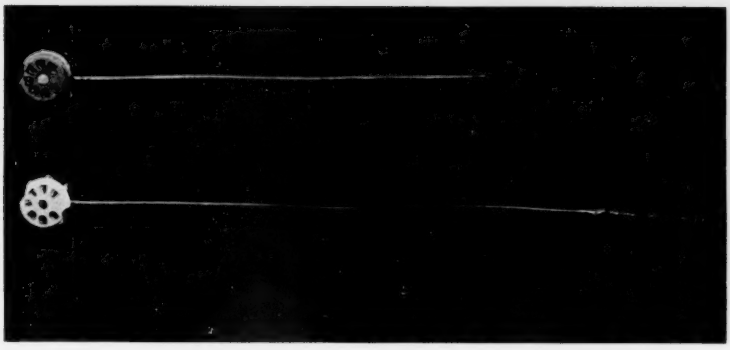

FIG. 1. Needle electrode (up) for studying the inside of the brain. It is formed by seven isolated wires with each tip 2 mm. apart from the others. The "needle" has a diameter of only 0.5 mm.

Plate electrode (down) for studying the surface of the brain. The "plate" holds the tips of the seven electrodes in place on the cerebral cortex.

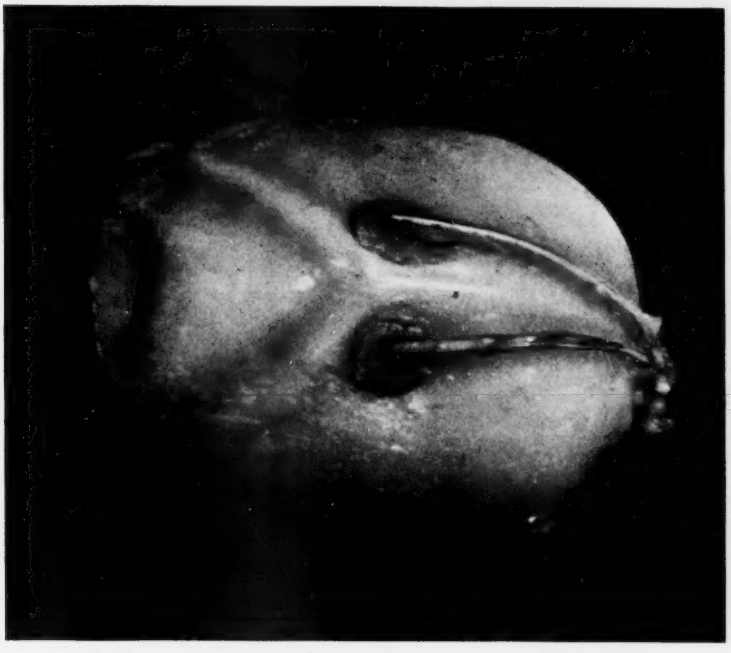

FIG. 2. Two groups of needle electrodes implanted in the nuclei of the amygdala of a squirrel monkey. Placed with the use of a Horsley-Clarke instrument, they are cemented to the bone and tied to the occipital protuberance.

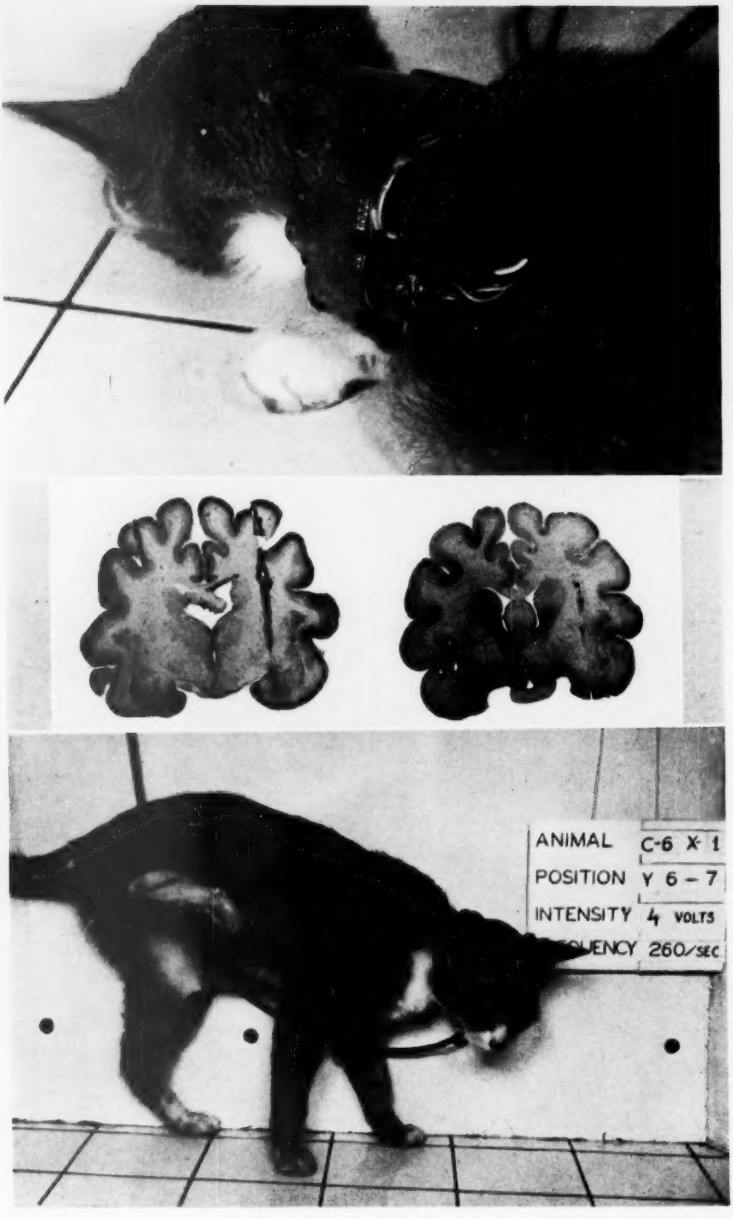

FIG. 3. Electrode slides beneath the skin of the neck and ends in a small female socket. Connection for stimulation, recording, or destruction is made by plugging in the special male socket.

FIG. 4. Path of the needle electrodes inside the brain. After months of implantation they are well tolerated. Only a small gliosis appears in the path of the electrodes.

FIG. 5. Stimulation of cortex buried inside the sulcus cruciatus in the conscious cat. A motor effect is evoked without emotional disturbance of the animal.

sary to make repeated observations of the functions of specific areas of the brain under varied conditions. The durability of the preparation permits its use in problems which require training, such as those encountered in psychology.

The avoidance of anesthesia has great value because drugs modify the excitability of the brain, and because during anesthesia sensitivity and normal activity are absent. Implanted electrodes make possible studies of behaviour, emotions, etc.

One of the greatest advantages of the method is the multiplicity of the electrodes. In general fourteen electrodes (two groups) or twenty-eight electrodes (four groups) have been used, but if necessary it is easy to increase the number (in some experiments I have used forty electrodes). The multiplicity of electrodes is especially useful for making correlations between different regions of the central nervous system.

By means of implanted electrodes the following operations may be performed: (a) stimulation, (b) recording, (c) making lesions by electrocoagulation, (d) recording of evoked potentials, and (e) all combinations of the preceding operations such as simultaneous or delayed stimulations of cerebral or cerebellar points, chronic stimulation (for example, one hour daily) of particular regions, etc.

The animal is connected to the recording or stimulating instruments by using a plastic-shielded, seven-wire cable ending in a male socket whose female equivalent is situated on the neck of the animal. Connection is made by merely plugging the sockets together. The system provides a good contact and is very convenient. The simplicity of the plugs overcomes the complexity of earlier techniques, one of which required the soldering and unsoldering of each wire for every experiment.

An animal accustomed to a collar and a plastic leash is unable to differentiate between his leash and the plastic cable and therefore does not know he is the object of attention. Thus inhibitory factors, so important in behaviour studies, are avoided. Freedom of movement during an experiment depends on the length of the plastic cable which, especially for stimulation, may be very long allowing unconstrained activity.

Usually experiments are conducted on a specially constructed stage (Fig. 5) that has good illumination, transparent windows, and a clearly legible adjustable sign on which can be indicated the day, hour, animal number, position of electrodes, and the parameters of stimulation. The stage arrangement is particularly useful for taking still and moving pictures and for making sound recordings. For stimulation the only necessary equipment is the stimulator, therefore it is easy to do experiments in any location, outside or inside the stage—an advantage since it prevents the animal from becoming conditioned to its surroundings. This is especially the case when an experiment involves repeated stimulations of areas dealing with emo-

tional behaviour (thalamus, hypothalamus), but it is not so important in the stimulation of the motor regions.

A well-known stereo technique is used for implantation of the needle electrode, exact placement at a predetermined point being easily accomplished. Histological study of the brain is necessary in order to check the exactitude of the position of the electrodes. In the plate electrodes a still picture and visual inspection will give the position of the electrodes. To know their precise location a continuous current is passed between electrodes 1 and 7 for five seconds to produce a small electrolytic lesion which can be found when the animal is sacrificed.

Two arguments may be presented against the technique. First, the presence of a foreign body inside the brain may disturb normal function. The importance of this factor is probably very small in the case of plate electrodes which produce no damage, but it is greater in the case of needle electrodes which produce a small but destructive lesion of brain tissue. After several months of implantation some gliosis appears around the needle electrodes. However, the extent of this gliosis is limited to about 0.5 mm. around the path of the needle. The histological aspect of the rest of the surrounding brain tissue is entirely normal.

Second, since the animals have spontaneous activity (motor and electrical), one must differentiate between normal activity and that artificially evoked by the experiment. Fortunately, since the experiments may be repeated as many times as necessary, it is possible to ascertain the difference. The spontaneous movements of the animal very appreciably complicate the electrical recording of the brain activity because of the artefacts produced by these movements. Differential amplification reduces artefacts, and in some experiments one must wait until the animal becomes inactive or sleepy. Since recording does not disturb the animal, it is easy to obtain good results. This technique has been tried successfully on various kinds of animals. Mice, cats, dogs, and monkeys with electrodes implanted in the frontal lobes (superficial and hidden cortex), lateral hypothalamus, amygdaloid nuclei, cingulate gyrus, cerebellum, etc., have been studied for periods of months. The presence of the electrodes did not disturb the health of the animals, and after regrowth of the hair shaved off for the operation it was impossible to differentiate between normal animals and those that had been operated upon.

Good biological tolerance of implanted electrodes and their successful use for local stimulation, recording, or destruction suggest the possibility of applying this technique to humans for diagnosis and therapy of cerebral disorders. This problem is at present under consideration.

Summary

A technique for implanting electrodes for long periods of time inside the brains of mice, cats, dogs, and monkeys has been described. Two kinds of

electrodes were presented: plate electrodes for the surface and needle electrodes for the interior of the brain. The first is placed under visual control, and the second by means of the Horsley-Clarke instrument. The particular advantages of this technique are as follows:

1. Large numbers of electrodes can be employed (14 to 40).

2. Several discrete points of the brain (surface and interior) can be studied individually or simultaneously.

3. Because of the shape and small size of the electrodes, there is no brain trauma with the use of the plate electrode, and a very limited amount of trauma in the case of the needle electrode (the diameter of the needle formed by seven electrodes is only 0.5 mm.).

4. It has been established that by employing biologically inert materials in the construction of the electrodes no tissue reaction is evoked by the plate electrode and only a very limited gliosis by the needle electrode.

5. Virtually all regions of the brain are made accessible for study.

6. The electrodes may remain implanted for long periods of time without causing any observable adverse effect upon the health or behaviour of the animal subjects. (Maximum period of observation one year.)

7. The electrodes may be employed for stimulation, recording, and production of lesions (electrocoagulation).

8. During experiments the animals have complete freedom of movement.

9. Connecting the animal to the electrical instruments is accomplished merely by plugging two sockets together at the neck of the animal.

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THE EFFECT OF HYDRAZINE DERIVATIVES OF ISONICOTINIC ACID ON THE INFECTION OF MICE WITH M. TUBERCULOSIS VAR. BOVIS*

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With the assistance of
D. ELDRIDGE, C. UNGER, AND E. TITSWORTH

While the first observations on the anti-tubercular activity of hydrazine derivatives of isonicotinic acid[5, 6] were made using the experimental infection of mice with Mycobacterium tuberculosis var. hominis, it has become evident through the work of Bernstein, Lott, Steinberg, and Yale[3] and Steenken and Wolinsky[11] that the bovine type of M. tuberculosis was also markedly sensitive to the new chemotherapeutic agents. The experiments with this organism that are being reported here supplement the work of Bernstein, *et al.*[2] inasmuch as the observation time was extended in an attempt to determine the duration of the protective effect brought about by the administration of the new compounds. Moreover, the lungs of the animals used in these experiments were examined and the bacteriological effect of the treatment was evaluated by culture. The observations made in this study are discussed in the present paper.

MATERIALS AND METHODS

The Ravenel strain of M. tuberculosis var. bovis, obtained through the courtesy of Dr. P. C. Eisman on June 18, 1951, was used in all the experiments.

In vitro and in vivo experiments. The techniques for evaluating the compounds *in vitro*[4] and *in vivo*[5] have been described before. In the latter experiments adult albino mice of 18-20 grams' weight originating from our colony were used. In addition to the experiments where animals infected intravenously were treated by medicated diet for 21 days and observed for an additional 21-day period, experiments were also carried out in which treatment by medicated diet was administered for a 28-day period and the animals then observed for an additional 72 days. Post-mortem examinations were carried out both on the animals that survived the observation period and also on those that succumbed during the experiments. Whenever possible, cultures of the lungs were made on egg-potato-medium[10] and histological sections taken.

EXPERIMENTAL RESULTS

In vitro experiments. The Ravenel strain of M. tuberculosis exhibited a sensitivity to Rimifon (isonicotinic acid hydrazide) and Marsilid (1-isonicotinyl-2-isopropylhydrazine) identical to that observed with the H37Rv strain,[4] namely, 0.015 mcg./ml. and 1.5 mcg./ml. respectively.

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In vivo experiments. As representative compounds to show the effect of the hydrazine derivatives of isonicotinic acid, Rimifon* and its isopropyl derivative Marsilid were selected. In some experiments, a third hydrazine derivative, namely, 1-isonicotinyl-2-d-glucosyl-hydrazine was included. The effect of this atoxic glucosyl derivative (LD_{50} for mice = greater than 2500 mg./kg. subcutaneously; 1750 mg./kg. by gavage) has been described by Zieper and Lewis[12] who employed this substance in a tuberculous monkey. Its activity in mice infected with the bovine strain of M. tuberculosis has also been studied in the experiments reported by Bernstein, *et al.*[2]

For reasons of comparison compounds of known anti-tubercular activity such as streptomycin, nicotinamide,[9] nicotinaldehyde thiosemicarbazone,[4, 7] and isonicotinaldehyde thiosemicarbazone[4, 8] were also included.

Protective experiments with a treatment period of 21 days and an observation period of an additional 21 days. In this group of experiments the intravenously infected mice were kept on a medicated diet for 21 days and then observed for an additional 21 days during which time they received a normal diet of Borden's Chunx. The hydrazine derivatives as well as streptomycin were tested at two dosage levels. The study with the two thiosemicarbazones was limited to the higher dose since previous experience had shown that no lasting effect could be expected in this series of compounds.[5] The results of the experiments are given in Table 1. This table does not contain information about the survival time, because all animals treated with the different drugs were still alive at the end of the 42-day observation period. The controls, on the other hand, had the usual average survival time of 22.1 days.

The data in Table 1, however, indicate that evaluation on the basis of survival time only, without post-mortem examination, does not give a true picture of the type of activity encountered in investigations of the anti-tubercular activity of new compounds. The column "average extent of lesions" which is based on the gross examination of the lungs clearly shows that at the end of the 21-day observation period following 21 days' treatment, anti-tubercular agents such as Rimifon and Marsilid still exerted a marked inhibition on the development of gross pulmonary lesions. This effect was found to be dependent on the dose inasmuch as the lower dose of 25.0 mg./kg./day allowed the development of somewhat more extensive pulmonary involvement. The other agents included in this series of experiments appeared considerably less active, particularly streptomycin. The lesions in the mice receiving streptomycin were as marked as those of the controls.

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* Rimifon, Marsilid, 1-isonicotinyl-2-d-glucosyl hydrazine, and isonicotinaldehyde thiosemicarbazone were synthesized by Dr. H. H. Fox, while nicotinaldehyde thiosemicarbazone was synthesized by Dr. T. S. Gardner and Mr. E. Wenis in the Roche Chemical Research Laboratories.

The thiosemicarbazones of nicotinaldehyde and isonicotinaldehyde appeared also inferior to the hydrazine derivatives of isonicotinic acid which is in agreement with earlier results.[5]

As a rule, the histological examination of the tissues reflected the gross findings inasmuch as the exudative lesions which dominate the microscopical appearance of the pulmonary tuberculosis of mice were considera-

TABLE 1

DEGREE OF PULMONARY INVOLVEMENT AND CULTURAL RESULTS OF MICE INFECTED INTRAVENOUSLY WITH M. TUBERCULOSIS VAR. BOVIS AND TREATED WITH RIMIFON, MARSILID, OR KNOWN ANTITUBERCULAR AGENTS

Infection: 0.5 ml. of a 10^{-1} dilution of a 7-10-day-old culture intravenously.
Treatment: Medicated diet 21 days. Normal diet 21 days.

Compound	*Dose mg./kg./day*	*Number of animals*	*Average extent of lesions*	*Number negative cultures / number animals cultured*
Rimifon	250.0	38	0.24[a]	28/28
Marsilid	250.0	18	0.22	15/18
Nicotinaldehyde thiosemicarbazone	250.0	18	1.94	3/13
Isonicotinaldehyde thiosemicarbazone	250.0	10	1.5	3/8
Streptomycin	150.0	20	2.75	9/20
Rimifon	25.0	36	0.75	28/33
Marsilid	25.0	20	1.1	13/17
Streptomycin	50.0	17	2.65	0/6
Controls	...	23	2.83	0/9

[a] 3.0 = 70-100% gross pulmonary involvement.
2.0 = 40-70% gross pulmonary involvement.
1.0 = 15-40% gross pulmonary involvement.

bly less marked in organs of animals treated with the hydrazine derivatives of isonicotinic acid. Complete lobular consolidation and even more extensive confluent lesions as observed in controls or unsuccessfully treated animals were absent in the protected mice, although very small scattered infiltrations were occasionally seen even in the lungs which appeared normal at gross examination.

Under these circumstances the results of the cultural examinations of the lungs seem to be of particular interest. The figures in the last column of Table 1 clearly demonstrate the fact that the hydrazine derivatives of isonicotinic acid and only these agents were able to prevent in a substantial number of animals the growth of M. tuberculosis in cultures taken 21 days

after discontinuance of treatment. The only anti-tubercular agent even approaching a similar effect is streptomycin if given in a high dose. It seems appropriate, however, to point out that the absence of growth by direct culturing of lung tissue may not represent a sufficiently sensitive method to allow any claims as to sterilization of the lung tissue by chemotherapy with the hydrazine derivatives of isonicotinic acid. As in our earlier observations of a similar nature[8] we can only claim that sufficient damage has been done to the organisms or their number so substantially reduced that recovery is no longer possible by cultural means.

TABLE 2

DEGREE OF PULMONARY INVOLVEMENT AND SURVIVAL TIME OF MICE INFECTED INTRAVENOUSLY WITH M. TUBERCULOSIS VAR. BOVIS AND TREATED WITH RIMIFON, ITS GLUCOSYL DERIVATIVE, STREPTOMYCIN, OR NICOTINAMIDE

Infection: 0.5 ml. of a 10^{-1} dilution of a 7-10-day-old culture intravenously.
Treatment: Medicated diet 28 days. Normal diet 72 days.

Compound	*Dose mg./kg./day*	*Number of animals*	*Number animals surviving*	*Average pulmonary involvement*[a]	*Average survival time: days*
Rimifon	250.0	15	12	2.0[b]	97.9 ± 5.0
Glucosyl	250.0	20	19	1.6	99.3 ± 0.15
Streptomycin	150.0	15	5	...	72.4 ± 24.2
Nicotinamide	2500.0	17	0	...	20.8 ± 4.6
Rimifon	25.0	20	20	1.9	100.0 ± 0.0
Glucosyl	25.0	19	2	3.4	53.5 ± 23.5
Streptomycin	50.0	18	0	...	48.8 ± 16.6
Controls	.....	20	0	3.8	21.7 ± 3.8

[a] Based on histological examination.
[b] 4.0 = 100% pulmonary involvement.
3.0 = 75% pulmonary involvement.
2.0 = 50% pulmonary involvement.
1.0 = 25% pulmonary involvement.

Protective experiments with a treatment period of 28 days and an observation period of an additional 72 days. Since the experiments described in the first section of this paper did not show a characteristic end-point of survival time, two groups of experiments were carried out in which the total observation time was extended to 100 days. The treatment period in these experiments was extended to 28 days instead of 21 days which leaves a lapse of 72 days between the termination of therapy and the final evaluation of the degree of protection.

In one of these experiments the usual intravenous infective dose of 0.5 ml. of a 10^{-1} dilution of a 7- to 10-day-old culture as employed in the earlier experiments was used. The results of these observations are given in Table 2. The average survival time as given in this table has been calcu-

lated, with the surviving animals being assigned a survival time figure of 100 days although they were actually sacrificed. The results of survival time are, therefore, supplemented by the number of surviving animals. This set of figures indicates that Rimifon if given in a high dose of 250 mg./kg./day as well as at the small dosage level of 25 mg./kg./day protected all or the majority of mice. It was interesting that the glucosyl derivative of Rimifon appeared fully active and comparable to Rimifon at 250 mg./kg./day, but at 25 mg./kg./day its effect dropped markedly and only 2 out of 19 animals survived the 100-day period. Streptomycin, even

TABLE 3

SURVIVAL TIME AND CULTURAL RESULTS OF MICE INFECTED INTRAVENOUSLY WITH M. TUBERCULOSIS VAR. BOVIS AND TREATED WITH RIMIFON, ITS GLUCOSYL DERIVATIVE, STREPTOMYCIN, OR NICOTINAMIDE

Infection: 0.5 ml. of a 7-10-day-old undiluted culture intravenously.
Treatment: Medicated diet 28 days. Normal diet 72 days.

Compound	*Dose mg./kg./day*	*Number of animals*	*Number animals surviving*	*Average survival time days*	*Number negative cultures* / *number animals cultured*
Rimifon	250.0	19	15	99.7 ± 15.9	9/12
Glucosyl	250.0	17	11	79.8 ± 28.2	2/8
Streptomycin	150.0	17	1	73.3 ± 13.2	....
Nicotinamide	2500.0	19	0	18.2 ± 4.5	....
Rimifon	25.0	12	10	95.3 ± 11.5	....
Glucosyl	25.0	20	0	46.3 ± 17.9	....
Streptomycin	50.0	16	2	64.3 ± 16.8	....
Controls	.....	20	0	17.2 ± 4.8	....

at a high dosage level of 150 mg./kg./day, was inferior to the hydrazine derivatives of isonicotinic acid. Nicotinamide failed to show any degree of protection although it always has a demonstrable effect in the infection with the human strain of M. tuberculosis.[4, 9] All surviving animals exhibited at gross as well as at microscopic examination lesions whose extent was, however, markedly reduced in all animals receiving Rimifon. A reduction of lesions was observed with the glucosyl derivative of Rimifon only at the high dosage level. Demonstration of tubercle bacilli in the sections of the lungs of these mice also showed that only a few scattered acid-fast organisms could be observed, while the lesions of the controls and unsuccessfully treated animals contained numerous bacilli in dense clusters.

In a second experiment presented in Table 3 a heavier infection was given, namely, 0.5 ml. of an undiluted culture, 7 to 10 days old, intravenously. Almost identical results were obtained as in the experiments just

described. The length of the average survival time as well as the number of animals surviving the entire period of observation was again most marked in case of Rimifon. Its glucosyl derivative appeared less active even at the high dosage level of 250 mg./kg./day. At the lower dose level, not only was the prolongation of life markedly reduced, but no final survivors were observed. Streptomycin in both dosages exhibited its well-known delay of death, but the number of final survivors was negligible. Nicotinamide, despite a high dose of 2500 mg./kg./day, was without effect in that the mice succumbed at about the same time as the controls.

Cultures, which were only taken from surviving animals, further emphasize the point that results based on survival time alone do not necessarily indicate the complete picture of anti-tubercular activity of closely related compounds. In the case of Rimifon an appreciable number of negative cultures were obtained from surviving animals, while with its glucosyl derivative growth of M. tuberculosis was observed in the majority of the cultures taken from the lungs.

Discussion

The data given in the experimental part of this paper confirm the value of the method of the fatal infection of mice with the Ravenel strain of M. tuberculosis var. bovis for the evaluation of the anti-tubercular activity of chemotherapeutic agents. The results also confirm the work of Bernstein, *et al.*[2] with the hydrazine derivatives of isonicotinic acid. Principally, the results obtained in these experiments with the Ravenel strain of M. tuberculosis var. bovis corroborate those described in earlier publications[5, 6] for M. tuberculosis H37Rv. Rimifon and Marsilid as the representative compounds of the hydrazine derivatives of isonicotinic acid not only considerably extended the survival time of the treated animals, but also produced almost the same reduction in the extent of lesions as described in experiments with M. tuberculosis var. hominis. Furthermore, the marked influence on the viability of M. tuberculosis var. hominis could also be demonstrated with the bovine type of M. tuberculosis even 72 days after discontinuation of treatment.

The entirety of these findings suggests that the determination of limited survival time as recommended by some authors[1, 3] does not always allow the necessary differentiation between chemically related compounds of different degrees of activity. The glucosyl derivative of Rimifon which appears under certain conditions equivalent to the parent substance can be shown to be inferior to Rimifon by the use of lower dosage ranges, by extended observation time, and by cultural means. In addition, the undoubtedly marked effect of streptomycin on the survival time loses its significance if one considers the number of final survivors at the end of the extended survival time and the presence of specific lesions. At the present time only Rimifon and the related Marsilid seem to satisfy the requirement for anti-tubercular drugs

which produce lasting protection apparently by their direct effect on the viability of the causative organisms.

Summary

1. The effect of hydrazine derivatives of isonicotinic acid in experimental infections with M. tuberculosis var. bovis as described by Steenken and Wolinsky and by Bernstein, *et al.* was confirmed in the present experiments in mice.

2. Additional evidence is presented to show that the new agents, e.g. Rimifon and Marsilid, exerted the same type of activity on the bovine type of strain as was earlier reported for infections with M. tuberculosis var. hominis. The oral administration of the compounds resulted in considerably prolonged survival time, absence or reduction of tuberculous lesions in the lungs, and prevention of growth of tubercle bacilli in cultures taken from the lungs.

3. The demonstration of these properties of the hydrazine derivatives of isonicotinic acid establishes their superiority to known anti-tubercular drugs and also allows the evaluation of the therapeutic potency of chemically closely related products.

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VENTRICULAR EXCITABILITY OF THE INTACT DOG HEART*

RAJINDER S. SIKAND,† LOUIS H. NAHUM, HERBERT LEVINE, AND HERMAN GELLER

Since the refractory period was first described by Fontana in 1760,[7] a variety of techniques has been employed to study the time-course of recovery of heart excitability and its relation to mechanical and electrical events during the cardiac cycle. Bowditch,[7] in 1871, stimulated the perfused apex of the frog heart with rhythmic shocks at intervals of 1, 2, 3, 4, or more seconds. He found that stimuli just strong enough to produce a response failed to do so regularly, and a response to each shock could be obtained only if the strength of the stimulus were considerably augmented. The greater the frequency of stimulation, the more marked was the irregularity of response. At faster rates a much stronger stimulus was required to produce regular responses. The threshold was found to diminish with successive responses.

Three years later, Kronecker and Stirling[7] performed similar experiments with an improved type of stimulator and were unable to demonstrate this irregularity. They found that if stimuli were of sufficient strength, each produced a response. Shocks of lesser intensity were without effect. They confirmed Bowditch's observation that there was a gradual diminution of threshold with successive responses.

Marey,[7] in 1876, studied a spontaneously beating frog heart stimulated by single shocks thrown in at random throughout the cardiac cycle. He demonstrated that a refractory period began at the onset of systole for each cycle. The duration of this refractory period depended on the intensity of stimulation and the condition of the preparation.

Trendelenburg,[16] in 1911, devised an ingenious delayer system which enabled him to apply stimuli at various points along the mechanical contraction curve of the spontaneously beating frog heart. He was the first to carry out a systematic study of the relationship between mechanical events and excitability. He demonstrated the presence of absolute and relative refractory periods, and related them to the components of the mechanical contraction curve.

The following year Trendelenburg[17] attempted the first correlation between excitability and electrical events, using the monophasic action

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potential of the isolated frog heart. He pointed out that it was sometimes possible to obtain a response on the descending limb of the monophasic action potential by means of a superthreshold stimulus. Threshold stimuli were generally not effective until one second after the end of the monophasic action potential, but they sometimes produced a response at its completion.

Mines,[10] in 1913, correlated the excitability of the spontaneously beating frog heart with a ventricular electrogram. He showed that the end of the refractory period corresponded with the termination of the electrical response.

Adrian's important study of the recovery process of excitable tissues[1, 2] included two groups of experiments bearing on the cardiac excitability cycle. In one group of experiments, frog hearts were perfused with fluids of different pH, and it was shown that when the pH was below seven, a supernormal period following the relative refractory period was developed. In the second group of experiments the relationship of excitability to the monophasic action potential of the frog heart was investigated. Pairs of induction shocks separated by a variable interval were employed, the first to drive the heart and the second to test its excitability. The second stimulus was four to five times the threshold strength. Results from a group of six experiments showed that the second shock was not effective until just after the complete disappearance of the monophasic electrical response. Adrian pointed out the necessity for allowing sufficient time to elapse between determinations to insure complete recovery from the effects of the previous stimulus.

The studies of Hoff and Nahum[8, 11] were the first to relate the excitability cycle to the electrocardiogram. Standard Limb Lead II was used. In experiments on cats, dogs, and chimpanzees the heart was exposed and testing stimuli were applied at random from a thyratron stimulator approximately one second apart. The relative refractory period occurred on the descending limb of the T wave and was often followed by a period of supernormality before excitability returned to threshold levels. However, there was no supernormality in unanesthetized decerebrate preparations, but it did occur in animals that were anesthetized with barbiturates. That the barbiturates were not responsible for the supernormal phase will be seen below.

Recently, an extensive investigation of the dog auricle and ventricle has been carried out using a method described by Suckling, *et al.*[18] In these studies the heart was exposed, the sinus node clamped off, and the heart driven at the desired rates. By means of a delayer circuit, testing stimuli of varying strengths and duration from a rectangular wave generator were applied during the entire cardiac cycle. The exposed heart was protected by a transparent artificial chest wall after the insertion of the driving, stimulating, and recording electrodes. Stimuli were applied every eight to

ten beats (every 2-5 seconds). The recovery of excitability in both auricle[9] and ventricle[16] was not a uniform process, and periods of hyperexcitability within the relative refractory period were demonstrated. The first[4] of these was shown to coincide with the vulnerable period for fibrillation described by Wiggers and Wegria.[18]

In the present study, the excitability cycle of the normal ventricle was investigated. It was considered advisable, therefore, to maintain an intact chest wall in order to avoid operative trauma and the loss of heat, CO_2, and

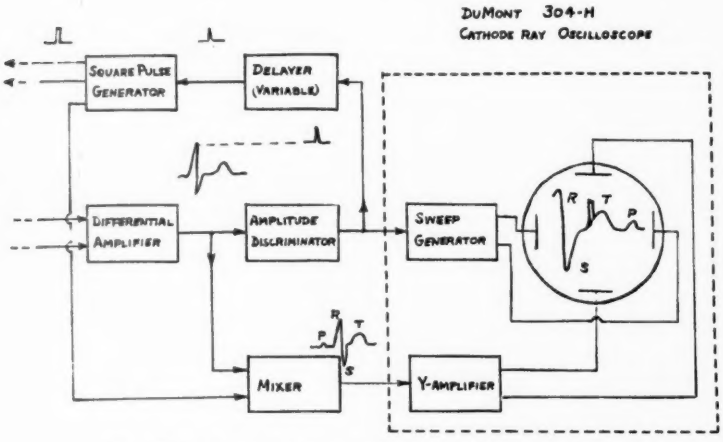

Fig. 1. Block diagram of apparatus used. See description in text.

moisture associated with exposure of the heart. Spontaneous rhythm was desired to preclude any alterations in excitability produced by the driving stimuli. Thus an attempt was made to evolve a method which would eliminate factors in the technique that in themselves alter excitability. After normal excitability under physiological circumstances had been established, information bearing on several important questions was obtained. The differences in the rates of recovery among various regions of the heart, as tentatively suggested by previous studies on the genesis of the T wave,[12] were confirmed. It was also possible to test the validity of the use of the QT interval as a measure of the duration of cardiac recovery.

Method

With these considerations in mind, the following technique was developed. Dogs were anesthetized with Nembutal (40 mgm./kgm.) administered intraperitoneally. As steady a state of anesthesia as possible was maintained throughout each experiment. Employing the Wilson central terminal, needle electrodes were used to record a "unipolar" precordial electrocardiogram over the left apex. As shown in Figure 1, this

FIG. 2. Stimulating electrode consisting of a 22-gauge needle through which runs a copper wire. Insulation extends to tip. Area of stimulation equals .5 sq. mm.

electrocardiogram was led to a differential R-C coupled amplifier, the output of which was fed to the rectangular wave generator through a variable delayer system. The amplitude discriminator of this delayer made it possible to utilize the peak of the R wave as a zero time base from which the testing stimulus could be delayed. A cathode-ray oscilloscope was used for monitoring purposes which made it possible to adjust the testing stimulus to any desired time in the cardiac cycle. The rectangular wave generator had a maximum output of 12 milliamperes. With a stimulating electrode in place, the current intensity was calibrated by measuring the voltage drop across a precision 1,000 ohm resistor inserted in one of the output leads. A mercury key was interposed between the generator and the concentric electrode. The concentric electrode is illustrated in Figure 2.

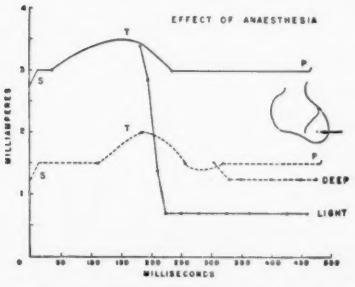

FIG. 3. Effect of varying levels of anesthesia upon excitability. Abscissa represents time in milliseconds in relation to electrocardiogram. Dotted lines represent excitability curve and electrocardiogram for deep anesthesia; solid lines represent excitability curve and electrocardiogram for light anesthesia. Ordinate represents minimum effective stimulus strength in milliamperes. Arrow indicates position of stimulating electrodes in both cases. See description in text.

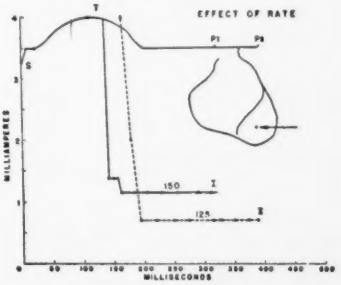

FIG. 4. Effect of variation in rate on excitability. Abscissa represents time in milliseconds in relation to electrocardiograms, which differ only in position of P waves. Ordinate represents minimum effective stimulus strength in milliamperes. Curve I obtained at rate of 150 and cycle length correspond with PI of electrocardiogram. Curve II obtained at rate of 125 and cycle length correspond with PII of electrocardiogram. Arrow indicates position of stimulating electrode. See description in text.

This electrode was inserted into a specific region of the myocardium through the intact chest wall. A cotton support minimized the movement of the external portion of the electrode. During the experiment, the position of the electrode was indicated by the configuration of the extrasystoles produced, and it was later confirmed by the autopsy. Testing stimuli were not applied more often than every two minutes for the reasons given below. The cycle was scanned at 5 millisecond intervals. A pulse duration of 0.5 milliseconds was used in these experiments.

RESULTS

In the course of these experiments, three factors were encountered which altered excitability and therefore had to be controlled before the true excitability cycle could be established.

1. *Anesthesia.* In many experiments it was observed that a sudden drop in threshold was an early indication of lightening anesthesia. The solid line

in Figure 3 illustrates this. The diastolic threshold period begins 15 milliseconds before the end of T, and the diastolic threshold value is 0.7 milliamperes. The dotted line represents the new excitability curve when the animal is given an additional dose of anesthetic. The diastolic threshold period then begins 10 milliseconds after the end of T and the threshold value rises to 1.35 milliamperes. Although the rate has not changed, the QT interval has lengthened from 245 milliseconds to 350 milliseconds and the T wave has become diphasic. The curve is shifted upwards and to the right. Therefore, anesthesia delays recovery, lengthens both refractory and QT intervals, and elevates the diastolic threshold.

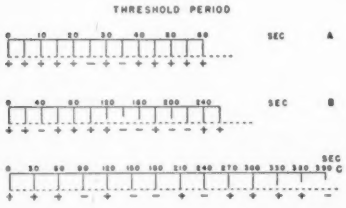

FIG. 5. Variations of threshold with frequent stimulation at the same point in the threshold period. A represents stimulation every 5 seconds, B stimulation every 20 seconds, and C stimulation every 30 seconds. Length of vertical lines indicates strength of stimulus applied; dotted line indicates original threshold level. Response indicated by plus, lack of response by minus. Notice elevation of threshold in A at 25 and 35 seconds, and lowering of threshold in B at 120 seconds, and in C at 330 and 360 seconds.

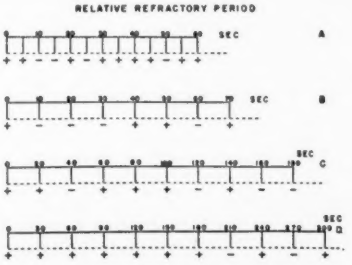

FIG. 6. Variations of threshold with frequent stimulation at the same point in the relative refractory period. A represents stimulation every 5 seconds, B every 10 seconds, C every 20 seconds, and D every 30 seconds. Length of vertical line indicates strength of stimulus applied; dotted line indicates original threshold level. Response indicated by plus, lack of response by minus. Note elevations of threshold at all points where minus sign appears.

2. *Heart rate.* Earlier investigations were carried out on hearts driven at specific rates. The present investigation was carried out on spontaneously beating hearts. It was observed that significant changes in excitability occurred when the rate varied spontaneously 20-30 beats per minute. Figure 4 shows curves obtained at rates of 125 and 150 beats per minute. At the faster rate, although the refractory period was shorter, the threshold values were higher. The threshold rose from 0.74 milliamperes at a rate of 125 beats per minute to 1.15 milliamperes at a rate of 150 beats per minute. The duration of the refractory period was shorter at a higher rate, but the ratio of the refractory period to cycle length remained unchanged.

3. *Frequency of testing stimuli.* The necessity for complete recovery from a testing stimulus has been recognized by previous investigators. Trendelenburg[16] and Lewis,[9] however, do not specify the time interval

allowed in their experiments. Adrian[2] permitted one minute to elapse between each testing stimulus, and Suckling, *et al.*,[15] stimulated every 8-10 beats (2-5 seconds). In our experiments, it has been found that there must be an interval of two minutes in order to obtain reproducible results. This two-minute period was determined by experiments such as those illustrated by Figures 5 and 6. Figure 5 shows threshold stimuli applied every 5, 20, and 30 seconds at a given point in the diastolic threshold period. Pronounced variations in response are apparent. In graph A, the increase in threshold value is indicated by failure of response at 25 and 35 seconds. This effect can be seen at 40 and 220 seconds in graph B, and at 90, 150, 180, and 240 seconds in graph C. Lowering of threshold value is manifested by response to subthreshold stimuli at 120 seconds in graph B, and 330 and 360 seconds in graph C. Similar changes are seen in Figure 6, which illustrates an identical experiment carried out at a point in the relative refractory period. Irregular failure of response may be seen in all four graphs.

Excitability Cycle at the Left Apical Region

A. *Animals with upright precordial T complex.* It should be emphasized that these results were reproducible in the same animal over long periods of time. The experiments that are reported are those in which the heart rates did not vary significantly, the depth of anesthesia was kept constant, and the testing shocks were applied no oftener than every two minutes. As the concentric electrode was inserted through the chest wall and directed towards the apex, the only justifiable assumption is that the points probed were in a region near the apex rather than exactly at the apex. Table 1 summarizes the 21 experiments in this series. Of these, 15 showed an upright precordial T wave. In 12 of the 15, the refractory period ended somewhere on the downstroke of T, i.e., 10-15 milliseconds before the end of T. In three others, the refractory period terminated at the end of T. The threshold in all these experiments varied from 0.35 milliamperes to 0.86 milliamperes (Fig. 7).

B. *Animals with diphasic precordial T complex.* In six experiments, the precordial T was diphasic and had an inverted terminal portion. In four of these, the refractory period was complete 10-35 milliseconds after the end of T, and in a fifth, it finished at the end of T. In the sixth, however, the refractory period terminated a little more than 50 milliseconds before the end of T. The threshold was in the same range as that found in the group with an upright T complex. A fairly constant correlation between the duration of the refractory period at the apex and the configuration of the precordial T was observed. Generally, when the T is upright, the refractory period ends on the downstroke of T, and when the T is diphasic and has a terminal inverted portion, the refractory period tends to finish after the end of T.

TABLE 1

EXCITABILITY AT LEFT APICAL REGION

Experiment no.	Heart rate per minute	Cycle length in msec.	Direction of precordial T. complex	QT in msec.	A.R.P. in msec.	T.R.P. in msec.	Beginning of D.T.P. before end of T in msec.	Beginning of D.T.P. after end of T in msec.	QT/RR in %	KQT	A.R.P./RR in %	R.R.P./RR in %	T.R.P./RR in %	Diastolic threshold values in mA.
2	133	450	upward	230	160	190	40		51%	.34	35.5	6.5	42.0	.80
3	125	480	"	245	195	235	10		51	.35	40.6	8.3	48.9	.69
5	125	480	"	265	215	245	20		55	.38	44.8	6.3	51	.69
*6	125	480	diphasic	340	310	350		10	62.5	.43	64.6	10.4	75	1.27
9	110	545	upward	280	220	230	50		50	.38	40.4	1.8	42.2	.35
11	135	444	"	220	160	190	30		50	.33	36	6.8	42.8	.86
12	135	444	diphasic	205	230	240		35	46	.31	51.8	2.3	54.1	.69
13	135	444	"	205	175	205	0	0	46	.31	39.5	6.8	46.0	.50
14	130	462	upward	240	210	225	15		52	.35	45.5	3.2	48.7	.50
16	158	380	"	210	115	210	0	0	55.3	.34	39.5	2.5	64.5	.69
18	109	550	"	305	255	305	0	0	55.5	.41	46.4	8.2	54.6	.85
20	137	438	"	245	190	210	35		56	.37	43.4	4.6	48	.86
22	120	500	diphasic	280	205	230	50		46	.40	41.0	5.0	46	.86
28	133	450	upward	315	190	270	45		70	.47	42.2	17.8	60	.86
30	107	560	diphasic	265	275	300		35	47	.39	49.2	4.5	53.7	.86
31	160	380	"	190	175	200		10	50	.31	46	6.6	52.6	.50
32	120	500	upward	270	210	295	25		54	.38	42	7	49	.70
34	136	440	"	240	170	195	45		54.5	.36	38.6	4.6	43.2	.69
4	150	400	"	235	185	205	30		59	.37	46.3	5	51.3	.86
7	105	572	"	285	220	230	55		50	.38	38.5	1.8	40.3	.86
27	92	650	"	305	235	305	0	0	46	.38	36.2	10.8	47	.86

* Deeper anesthesia.

A.R.P. = Absolute refractory period.
R.R.P. = Relative refractory period.
RR = Cycle length.
T.R.P. = Total refractory period.
D.T.P. = Diastolic threshold period.

Excitability Cycle at Intermediate Points on the Left Ventricle Between Apex and Base

Of seven experiments (Table 2), six exhibited an upright precordial T, and in five of these the refractory period ended 20 to 75 milliseconds before the end of T. In one, the refractory period terminated at the end of T. In the experiment with a diphasic T, the refractory period also terminated before the end of T. In these experiments, however, the range of diastolic threshold was between 1.10 milliamperes to 1.64 milliamperes. The significant change between the apex and the intermediate points was the elevation of the diastolic threshold values. Figure 8 illustrates this series.

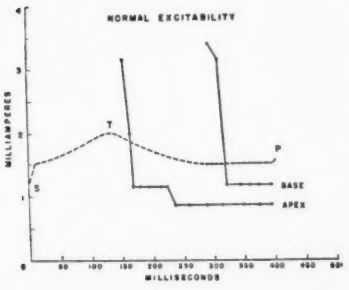

FIG. 7. Normal excitability at left apex and left base. Abscissa represents time in milliseconds in relation to electrocardiogram represented by dotted line. Ordinate represents minimal effective stimulus strength in milliamperes. See description in text.

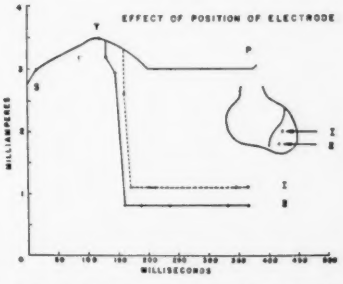

FIG. 8. Effect of change in electrode position on excitability. Abscissa represents time in milliseconds in relation to electrocardiogram. Ordinate represents minimum effective stimulus strength in milliamperes. Arrows indicate position of electrode for respective excitability curves. See description in text.

The Excitability Cycle at the Base of the Intact Heart

(a) *Right base.* Six determinations were carried out at the base of the right ventricle. In each experiment, the T wave was upright in the precordial leads. In four of these experiments, the refractory period finished 10 to 110 milliseconds after the end of T, in one at the end of T, and in one other 10 milliseconds before the end of T. Therefore, with an upright T in the precordial leads, the majority of instances showed that the refractory period was completed after the end of T (Fig. 7).

(b) *Left base.* Seven experiments were carried out with the electrode at the base of the left ventricle. In five experiments, the T wave was upright in the precordial leads, and in four of these five, the refractory period terminated well beyond the end of T. In two of the seven, the precordial T was inverted, and in both of these the refractory period concluded before the end of T. The diastolic threshold levels were about the same as in those

TABLE 2

EXCITABILITY AT THE LEFT INTERMEDIATE REGIONS

Experiment no.	*Heart rate per minute*	*Cycle length in msec.*	*Direction of precordial T. complex*	*QT in msec.*	*A.R.P. in msec.*	*T.R.P. in msec.*	*Beginning of D.T.P. before end of T in msec.*	*Beginning of D.T.P. after end of T in msec.*	*QT/RR in %*	*KQT*	*A.R.P./RR in %*	*R.R.P./RR in %*	*T.R.P./RR in %*	*Diastolic threshold values in mA.*
2(b)	133	450	upward	240	190	210	30		51%	.34			44.4	1.25
8	150	400	"	250	160	210	40		62.5	.40	40	13.8	53.8	1.64
19	88	680	"	360	300	340	20	—	53	.44	44.2	5.9	48.1	1.3
36	167	359	"	200	170	200	0	0	56	.34	47.4	8.4	54.8	1.37
37	150	400	"	220	185	200	20	—	52.5	.33	46.3	3.7	48	1.14
39	60	1000	diphasic	560	410	490	70	—	56	.56	41	8	49	1.17
40	94	638	upward	375	285	300	75	—	59	.47	44.7	2.3	47	1.5

experiments where the electrodes were located at the intermediate points and at the right ventricular base, i.e., varying from 1.15 to 2.18 milliamperes.

In general, there was a close correlation between the direction of the precordial T wave and the duration of the refractory period of the base. When the T wave was inverted, the refractory period tended to end before the completion of T, and when the T was upright, the refractory period terminated beyond the end of T.

Absolute Refractory Period

The duration of the absolute refractory period varied in different regions of the heart, being shortest at the apical region and longest at the basal regions of the ventricle, which may be seen in experiment No. 34, Table 1, and experiment No. 35, Table 3. In different animals with the same rate, the absolute refractory periods approximated each other except for an occasional wide variation, as for example Nos. 11, 12, 13, and 14 in Table 1 and Nos. 24 and 25 in Table 3. Similarly, some observations at faster rates showed absolute refractory periods that were longer than those obtained at slower rates as demonstrated in experiments Nos. 8 and 36 in Table 2.

There was no fixed ratio between cycle length and the absolute refractory period, although in general the latter shortened as the cycle shortened, and lengthened as the cycle lengthened.

Relative Refractory Period

The relative refractory period was extremely variable, having durations from about 5-10 milliseconds to 100 milliseconds. It possessed no fixed ratio to that of the absolute refractory period since it ranged from 1 : 3 to 1 : 26. The latter value was found at the base. In 30 of the 41 experiments the relative refractory period showed a progressive decline towards the diastolic threshold without exhibiting any of the oscillations described by Brooks and his associates. However, in 11 experiments plateaus but not "dips" occurred sometimes in the first and at other times in the last portion of the relative refractory period. Because the plateaus had no definite location in the relative refractory periods, one might suspect them to be artefacts, especially since in the large majority of experiments plateaus were not encountered. A possible explanation of their genesis is presented below.

Total Refractory Period

The total refractory period varied in different regions of the heart, and its duration was not synonymous with the QT interval as measured by the precordial electrocardiogram. With an upright precordial T complex the total refractory period of the apical region was shorter than the QT, and at the base it was longer than the QT. With an inverted precordial T complex

TABLE 3

EXCITABILITY OF THE LEFT BASAL REGIONS

Experiment no.	Heart rate per minute	Cycle length in msec.	Direction of precordial T. complex	QT in msec.	A.R.P. in msec.	T.R.P. in msec.	Beginning of D.T.P. before end of T in msec.	Beginning of D.T.P. after end of T in msec.	QT/RR in %	KQT	A.R.P./RR in %	R.R.P./RR in %	T.R.P./RR in %	Diastolic threshold values in mA.
1	120	500	downward	320	210	235	85	—	64	.45	42	5	47	1.14
10	150	400	"	210	180	205	5	—	52.5	.33	45	6.3	51.3	1.14
17	120	500	upward	260	235	270	—	10	52	.35	47	7	54	1.15
21	154	390	"	200	175	210	—	10	51.3	.32	45	9	54	1.2
24	167	360	"	205	230	235	—	30	57	.34	63.8	2.8	66.6	
25	167	360	"	215	185	200	15	—	58	.36	51.4	4.2	55.6	1.14
35	136	440	"	200	202	210	—	10	54.5	.36	46	1.6	47.6	1.14

TABLE 4

EXCITABILITY OF THE RIGHT VENTRICLE

Experiment no.	Heart rate per minute	Cycle length in msec.	Direction of precordial T. complex	QT in msec.	A.R.P. in msec.	T.R.P. in msec.	Beginning of D.T.P. before end of T in msec.	Beginning of D.T.P. after end of T in msec.	QT/RR in %	KQT	A.R.P./RR in %	R.R.P./RR in %	T.R.P./RR in %	Diastolic threshold values in mA.
15	120	500	upward	280	330	355	—	75	56	.40	66	5	71	1.18
23	125	480	"	220	210	220	0	0	46	.32	43.8	2.1	45.9	1.14
26	92	650	"	300	300	410	0	110	46	.37	46.2	16.9	63.1	1.14
29	100	600	"	270	295	295	0	25	45	.35	49.2	0	49.2	.69
33	150	400	"	210	185	220	0	10	52.5	.33	46.3	7.5	53.8	1.18
38	143	420	"	230	190	220	10	—	54.7	.36	45.2	7.2	52.4	1.27

the conditions are exactly reversed. The absolute refractory period of the apical region was longer than the QT and at the base it was shorter than the QT. Thus a fairly constant relationship was found between the duration of the total refractory period of the different regions of the heart, the configuration of the precordial T complex, and the QT.

Discussion

Results suggest that the direction of the precordial T wave is the clue to the duration of the total refractory period at the apex or at the base in relation to the QT interval. When the T was upright, the refractory period of the apical region terminated in most instances on its downstroke and occasionally at its end, whereas at the basal regions, the refractory period terminated at the end of T or well beyond it. In other words, the apical regions recovered earlier than the basal regions when the T wave was upright. When the precordial T complex was inverted the total refractory period of the apical region was longer than that at the base. Under such conditions the recovery of the basal regions occurred earlier than that of the apical regions of the ventricle. A correlation between the speed of recovery and the configuration of the T complex, either at the apex or at the base, has been shown by another method.[12] Acceleration of recovery by heating the apex produced an upright precordial T, whereas slowing of recovery by cooling the apex caused inversion of T. Man and the normal dog exhibit an upright T in the left precordial lead, and for this reason it was suggested that the apex repolarizes faster than the base and completes its recovery sooner. The investigations reported here lend support to the above view since the apex actually had a shorter total refractory period than the base in those experiments in which the precordial T was upright.

The QT interval in the electrocardiogram is considered to represent the duration of excitation and recovery of the ventricles. It has already been pointed out,[5] however, that QT in any one lead does not exactly reflect the "true QT" period. That must be obtained from multiple simultaneously recorded electrocardiograms measured from the earliest onset of Q to the latest termination of T. In these experiments the correlation of the refractory period was established with the precordial electrocardiogram, for it was found that this lead most closely approximated the "absolute QT" as obtained from multiple simultaneously recorded electrocardiograms. These studies showed that the total refractory period, either at the apex or at the base, did not correspond with the duration of QT. In the majority of instances the longest refractory period extended well beyond the end of T. It is obvious, therefore, that the period of excitation and recovery of the ventricles is not represented by the QT. It has been proposed that QU instead of QT might properly represent the period of electrical systole. It can be seen from the figures reported in the tables that the total refractory period does not end at a specific point at or beyond T, but that it varies in

different regions of the heart and in different hearts. Hecht's[6] summary of QT duration in several diseased conditions showed random changes. The disagreement among clinicians as to the significance of QT changes in disease may exist because of a failure to realize that (a) ordinary QT measurements in single leads are misleading, and (b) that QT itself is not correlated with the termination of recovery in all regions of the heart. If the "true QT" does not measure the period of excitation and recovery, it is clear that the method of averaging the various QT's in all leads would produce a shorter value and make it even less significant.

In all experiments the diastolic threshold was found to be a smooth plateau which began at the end of the relative refractory period. In no instance was supernormality encountered. However, response from a subthreshold stimulus can be produced when testing stimuli are applied 5-10 seconds apart (Fig. 8). The occurrence of supernormality has also been observed when the pH turns to the acid side,[1] and in an open chest preparation with barbiturate anesthesia.[8] Apparently, it does not occur if the heart remains in its normal environment and if the testing stimuli are applied no oftener than every two minutes.

The diastolic threshold remained a smooth plateau regardless of the region studied. However, the threshold levels changed from region to region, being lowest at the apex and highest at the base. The factors or processes that determine this gradient in the diastolic threshold levels for the different regions of the heart are not known, though it has been shown[18] that a temperature differential exists between the apex and the base. The apex is warmer by 0.25 to 0.5° C. It may well be that the higher temperature at the apex is one of the operative mechanisms responsible for the variations in diastolic threshold levels between apex and base.

These studies also showed that the diastolic threshold depended upon (a) rate and (b) depth of anesthesia. Increase in rate from 20-25 beats per minute was accompanied by an increase in the diastolic threshold value. Therefore the rise in diastolic threshold that accompanies significant increase in rate may be involved in the mechanisms operative in the spontaneous termination of paroxysmal tachycardias. If the elevation of threshold were to continue progressively as the rate accelerated, it might be expected finally to reach a level too high for depolarization by the "inner stimulus." In such an event the heart could stop for a variable period and only start again when the threshold falls enough to permit "autogenous excitation."

We have already mentioned our suspicion that the plateau in the relative refractory period, which occurred in a quarter of the experiments, may have been due to threshold shifts caused by changes in the level of anesthesia. If anesthesia lightened during this period, a drop in threshold would flatten the declining curve. This explains the random occurrence of the plateau as well as its infrequency in different parts of the relative refractory

period. However, the large majority of our experiments showed a smooth and progressive drop in threshold.

The direct action of the anesthesia upon the myocardial cell is established by these experiments, since the increase in the depth of anesthesia elevated the diastolic threshold values. This effect upon excitability furnishes an explanation for the effectiveness of the use of barbiturates for the abolition of ectopic rhythms.

Summary

1. The excitability cycle of apical, intermediate, and basal regions of the dog heart was established and correlated with the surface potential, i.e., left apical "unipolar" precordial electrocardiogram, of the intact animal.

2. The total refractory period did not correspond with the QT interval.

3. With an upright precordial T complex, the total refractory period was shorter at the apex than at the base. However, with an inverted precordial T, the total refractory period was longer at the apex than at the base.

4. The diastolic threshold period was a plateau in all experiments except when testing stimuli were applied more often than every 2 minutes.

5. The relative refractory period was a straight line graph.

6. The diastolic threshold was lowest at the apical region and highest at the basal region of the ventricles.

7. The threshold fell with lightening anesthesia and vice versa.

8. Increased heart rate was accompanied by elevated threshold.

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"SAPROPHYTIC FORM" OF *HISTOPLASMA CAPSULATUM IN VIVO**

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It is the purpose of this paper to report a unique observation concerning *Histoplasma capsulatum*. This diphasic fungus, according to previous reports, produces only yeast-like forms in the body of man and other animals. In culture, either the mycelial stage or the yeast-like form is obtained according to the experimental conditions. For the first time, so far as the author is aware, the mycelial stage, considered to be characteristic of the saprophytic portion of the life cycle, has been encountered in animals.

If tissue containing *Histoplasma capsulatum* is carefully macerated in a tissue grinder and then implanted on Sabouraud's medium and incubated at room temperature, the filamentous form of this organism develops. The mycelium, at first white, gradually becomes brownish and may produce pyriform conidia, but, as the culture ages, there also will be produced many thick-walled tuberculated chlamydospores. The production of tuberculated chlamydospores makes the identification of *H. capsulatum* certain among the fungi pathogenic to man and animal. If, however, macerated tissue is planted on fresh, moist blood agar in tubes sealed with paraffin, and the tubes are then incubated at 37° C., the yeast-like form will develop.

When the yeast form of *Histoplasma capsulatum* is injected into experimental animals, the cells of the reticulo-endothelial system are induced to proliferate. The mononuclear cells are literally packed with the yeast-like organisms. In the liver such proliferation of macrophages may give rise to focal lesions which undergo necrosis and involve adjacent liver parenchyma. If the animals are injected with a saline suspension of the filamentous form of this organism, their tissues at the time of death or autopsy will contain the yeast form.

While examining tissues of animals experimentally infected with the yeast form of *H. capsulatum*, a necrotic lesion in the liver of one of these animals was noted to contain masses of hyphae and many tuberculated chlamydospores. For reasons to be stated, however, it is not at present possible to offer any explanation for this reversion *in vivo* of the parasitic form of this fungus to the mycelial phase.

To obtain tissue infected with *H. capsulatum* for teaching purposes, eight white mice of unknown strain, weighing between 16 and 20 grams, were

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inoculated intraperitoneally with 1 ml. of a saline suspension containing 0.02 ml. of the yeast form of the organism.* Thirty-two days later all were alive although several showed evidence of being ill, as indicated by loss of weight, ruffled hair, and listlessness. All were killed with illuminating gas and promptly autopsied. No attempt was made to record the gross pathology of each mouse or to keep separate for histologic studies the tissues of each animal. The spleens of all of the mice were greatly enlarged. Five of them had moderately enlarged, pale livers. One liver was very friable and reduced in size. The kidneys of two mice were pale but without gross lesions. Livers of three mice, including the friable one, along with spleens, lungs, and a pair of the pale kidneys, were placed into a specimen jar containing 10% formalin. Care was taken to submerge the tissues completely. Four days later the tissues were embedded in paraffin and slides made for histologic studies, three slides being made from each block of tissue. One slide was stained with hematoxylin and eosin, while the other two were stained by the modified Hotchkiss-McManus method for fungi.

Examination of all tissues, with the exception of one specimen of liver, revealed the presence of the yeast form of *Histoplasma capsulatum*. This liver tissue was seen to contain a large necrotic area which erupted to the surface, in which were many hyphae and tuberculated chlamydospores (Figs. 1, 2, 3, 4). The yeast form of this fungus was not seen in the necrotic area, and only after very careful examination of the remainder of the tissue was it possible to find any yeast forms. The filamentous form was not found in the other two livers nor in any of the other tissues.

Cultures of the pooled tissues, made at the same time that the tissues were placed in formalin, were positive for *H. capsulatum*.

Discussion

The manner in which these tissues were prepared does not permit any explanation for the reversion of the yeast form of this strain† of *Histoplasma capsulatum* to the mycelial phase *in vivo*. Of the three livers examined, only one showed an area of necrosis, and it was here that the hyphae and chlamydospores were seen. In previous experiments with this same strain of *H. capsulatum*, however, the author has seen small necrotic areas in liver of infected animals which contained the yeast form of this organism, but never the filamentous form. Tager and Liebow[3] and Parsons,[1] as well as other investigators, using the filamentous form of the fungus were able to produce experimental histoplasmosis in mice, but the tissues revealed only the yeast form of the organism.

To account for the observation described in this paper would require consideration of certain factors, including the nutritional state of the mice

* Microscopic examination of this suspension revealed nothing but the yeast form; no hyphae were seen.

† Strain G-16, Army Medical School, Washington, D. C.

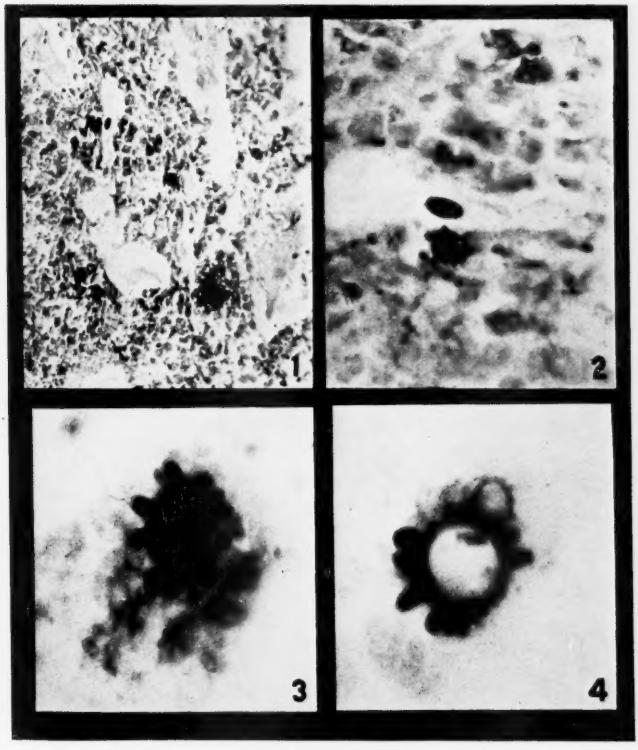

FIG. 1. Hotchkiss-McManus stain of the necrotic lesion in the liver, showing a mass of hyphae and five tuberculated chlamydospores. x 475.

FIG. 2. A tuberculated chlamydospore. Note the hyphae also present in the tissue. x 1470.

FIG. 3. Surface view of one of the tuberculated chlamydospores. x 2700.

FIG. 4. Optical section of a tuberculated chlamydospore. x 2700.

when inoculated and during the course of infection, the presence or absence of other diseases, the strain of mice used, the sex, age, and weight, the route of inoculation, the amount of inoculum and the method of its preparation. The growth characteristics of the particular strain of *H. capsulatum* would also have to be investigated.

The finding of the filamentous form in the liver of this infected mouse has instigated a series of studies on the entire subject of the dimorphism of human pathogenic fungi.

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THE BIOCHEMICAL TARGET OF FLEXNER DYSENTERY SOMATIC ANTIGEN

Studies on the Rat Using Antigen Labeled with I^{131}*

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The effect of various harmful substances on proteins of living cells and the response of the proteins to stimulus from such compounds are of interest in many biological problems, such as growth, immunity, disease processes, etc. The little-known stimulus and response phenomena in this field could be investigated if there were available some specific system composed of a purified protein as the target on the one hand and of a known harmful agent on the other.

Certain bacterial extracts and purified toxins have been shown to produce symptoms of disease upon injection of milligram or even microgram amounts in animals and man. In the case of some of these toxins it is possible that the chain of events leading to displacement of the body from the stable to the diseased state is initiated to some extent by direct, specific inhibition of some enzyme system. As part of the reaction to such an effect it seems possible, in view of the normal rapid process of regeneration which proteins undergo, that the regenerative processes in those enzyme systems injured by a toxin play a rôle in the production of immunity.

The possible rôle of such enzymes in the development of the diseased state and of immunity could be investigated with an appropriate system composed of purified antigenic toxin and its biochemical target in the host. However, very little is known of specific effects of antigenic toxins in disease. As a first step in finding a measurable system, we have sought the physiological target of Flexner dysentery somatic antigen by measuring the radioactivity present in various organs of the rat after administration of a purified preparation of the antigen labeled with I^{131}. By seeking a target we wish to approach the problem simply and without assumptions as to the mechanism of infection and disease. We wish to describe as far as possible what this toxic antigenic material does in the host. "Target" refers to the system of receptors, whatever they may be.

Critical analysis of present understanding[7, 9, 10, 12, 16–19, 21] regarding the loci of the interaction of this toxic antigen with cells permits no definite conclu-

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sions other than (a) the possibility that some cells of the colon are the ones to suffer a primary invasion and are especially susceptible to the toxin and (b) indications of a direct toxic effect on other tissues are minimal in reports made so far. Isotopes have been used previously to label antigens and antibodies.[11a]

Approach and Limitations of the Experiments

In approaching this problem one major consideration is that of adequate purity of the toxin-antigen, since appreciable amounts of impurity, if capable of diazotization, could lead to serious errors in the distribution data. To meet this as far as possible two different antigen preparations were used, each obtained by a separate method involving a number of steps in purification. One gave partly particulate aqueous solutions, the other, opalescent clear ones.

It is also important that the label be attached to the purified toxin-antigen in firm linkage and only in such form; otherwise, one could not follow the fate of the labeled material accurately. To accomplish this purpose, a synthesis on a micro scale had to be developed for p-iodoaniline of which the iodine was composed in part of I^{131}. This substance was isolated in pure form and was diazotized to the antigen under conditions controlled by first treating horse serum globulin and also antigen with non-radioactive p-iodoaniline. To remove excess reagent the product was dialyzed thoroughly, after which it was found to have taken up 55% of the amount of p-iodoaniline calculated on the basis of the reported tyrosine content of 8% by weight,[35] the tyrosine presumably being part of a polypeptide moiety of the antigen molecule.

Stability of the label on the antigen was tested in the fifth experiment in which the animal was sacrificed after 24 hours. Liver and blood were dialyzed for 36 hours with no radioactivity appearing in the dialysate. Some of the labeled antigen, of which part was given to the rat, was dialyzed similarly at this time with the appearance of 6.5% of its activity in the dialysate. Also a sample of the urine from the sixth (72 hours) experiment was dialyzed in the same way, 50% of the activity being found in the dialysate. The latter finding suggests that some of the material may be split in the process of excretion although bacterial action cannot be excluded. Landsteiner[11] has provided ample evidence of stability of the diazo-linked antigen, at least for as long as required to stimulate production of antibodies. Stability of the iodine is demonstrated by the complete recovery of activity in the organs and the urine (Tables 4 and 6). If iodine had been freed, much of it should have found its way to the thyroid gland and this would not have been recovered in the urine and organs isolated.

The results would be of doubtful significance if the toxicity of the labeled material were appreciably different from that of the unlabeled antigen, or if the animals were not sacrificed near the time of maximum toxicity. The

latter objective was sought by observation and, for Experiment #1, also by analogy with a large number of toxicity studies in rats and mice. Evidence for identity of toxicity of the labeled and unlabeled material was obtained by finding that symptoms and degree of weight loss were indistinguishable between the natural and the labeled toxin. The interpretation of the results is also dependent on a dose being given that is just enough to make the animal moderately ill. Since this was done, all of the antigen given to each rat can be considered necessary to the diseased state.

Justification for applying to the natural disease information derived from studies employing certain chemical substances has come from experimental work[3, 4, 9, 10, 13, 16] showing that the symptoms of dysentery can be reproduced in animals by extracts, in the absence of live bacteria. This justification was further substantiated by a large number of toxicity tests on mice[1] in which identity of symptoms was observed both in the experimental infection and after injection of a purified antigen of Flexner bacilli.

In six different experiments the toxin-antigen was given by various modes of administration to rats in doses so chosen that moderate definite signs of toxicity appeared. After a suitable period the animals were killed by ether inhalation, the organs removed promptly and their radioactivity measured. As the work was so exploratory in nature, the aim of each of the six experiments was based on the results of previous ones.

Results

The first experiment was performed in order to obtain orientation regarding distribution of the toxin; 4.75 mg. of the partly particulate antigen A, labeled with I^{131}, and containing a total measured activity of 15.5×10^2 c/s,* were given to one 100 g. rat intraperitoneally, as done routinely in toxicity tests. After 18 hours, although the animal looked quite well, he had lost 5.5 g. of weight; for several hours after the second hour he showed mild signs of weakness, irritability, ruffled hair, unwillingness to move, and rapid, shallow respirations. Previous experience with the antigen in mice and rats showed that the rat probably was experiencing a maximal toxic effect up to 18 hours and therefore it was sacrificed at this time.

On opening the peritoneal cavity the orange color of the azoantigen was visible in several areas in the mesentery, an appreciable fraction of the injected substance having been unabsorbed. A piece of mesentery that showed minimal evidence of orange-colored material was removed and found to have a specific radioactivity almost five-fold greater than that of any other tissue isolated (Table 1). Of the total antigen administered only 4.4% was found in the organs isolated (including the blood), and 5.5% was excreted in the urine.

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* Counts per second.

Recovery in the second experiment (Table 2), where the antigen was given intravenously, shows that the few tissues not isolated in this first experiment, such as bone and skin, could have taken up no more than an amount of antigen equivalent to that in the organs isolated. Therefore,

TABLE 1

INTRAPERITONEAL INJECTION OF 4.75 MG. OF DYSENTERY ANTIGEN LABELED WITH I^{131} INTO 100 G. RAT SACRIFICED AFTER 18 HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*Total antigen*	*100 × ratio of blood to tissue concentration*
	mg.	*Counts/min.*	*μg. antigen per g. tissue*	*μg.*	*Per cent*
Mesentery	309	5000	551	....	....
Spleen	70	260	121	38	3.5
Stomach	306	175	22	25	19
Adrenal	45.5	18.0	12	0.5	35
Liver	660	99.0	6.8	33	62
Thymus region*	73	15.0	6.3	1	67
Kidney	229	33.0	5.5	6	76
Abdominal muscle	77	12.5	5.1	5	82
Upper ileum	114	18.5	5.1	3	82
Blood	1700	81.5	4.2	21	100
Pancreas	223	27.5	4.1	1	102
Lower ileum	106	14.0	4.1	3	102
Duodenum	98	12.0	3.8	7	111
Lungs	156	18.0	3.7	3	113
Heart	109	11.0	3.1	1	135
Colon	212	12.5	2.2	1	190
Testis and epididymis	289	16.0	1.9	2	220
Leg muscle	419	15.0	1.3	°56	320
Brain	362	none	0	0	....
Total in organs				207	
Urine	2000	6.24×10^9	127	260	3.3

* Retrosternal tissue in region of thymus was dissected out.
° Total in all voluntary muscle, assuming weight of latter is equal to 40% of the weight of the whole animal.

something of the order of 85% of the administered dose must have remained unabsorbed in the peritoneal cavity when the animal was sacrificed.

The finding of a large fraction of the injected dose remaining in the peritoneal cavity indicates that the portal of entry may play an important rôle in the entrance of toxic chemical substances to body tissues, a subject about which little is known in the case of bacterial toxins. One would expect from this finding that the toxic effect would be very much greater when the antigen was given intravenously, provided that toxicity were proportional

to concentration in body fluids at any given time. Verification of this came in later experiments in which 5% of the dose given here was injected intravenously, immediately producing signs of marked toxicity. Also, extreme increases in toxicity were observed in humans by Cooper and Tepper,[5] after

TABLE 2

INJECTION OF 0.25 MG. OF LABELED DYSENTERY ANTIGEN INTO THE FEMORAL VEIN OF A 100 G. RAT SACRIFICED AFTER FOUR HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*Total antigen*	*100 × ratio of blood to tissue concentration*
	mg.	*Counts/min.*	*μg. antigen per g. tissue*	*μg.*	*Per cent*
Lungs	150	202	23	19	7.4
Liver	301	221	14	78	12
Lower ileum	246	67.2	5.0	10	34
Spleen	166	31.8	3.4	5	50
Kidney	226	34.2	2.7	3	63
Blood	3000	81.6	1.7	8.5	100
Stomach	146	10.2	1.2	0.9	140
Lymph nodes	62	3.0	0.7	<0.1	240
Mesentery	138	4.8	0.6	0.1	280
Upper ileum	242	7.2	0.5	0.6	340
Pancreas	165	3.0	0.4	0.1	420
Colon	435	4.8	0.2	0.3	850
Leg muscle	355	3.6	0.2	[a]9	850
Duodenum	150	0.6	....	....	.....
Heart	99	0.6	....	....	.....
Testis	264	0.0	....	....	.....
Adrenal	27	0.0	....	....	.....
Brain	384	0.0	....	....	.....
Epididymis	186	0.0	....	....	.....
Total in organs				135	

[a] Total in all voluntary muscle, assuming weight of latter is equal to 40% of the weight of the whole animal.

giving intravenously vaccines made from the whole Flexner dysentery, Type III, Z, organisms.

Numerous studies on mice[1,3] have shown that 18 to 20 hours after intraperitoneal injection of Flexner antigen preparations the signs of toxicity diminish rapidly. In the rat, also, it was found by preliminary tests that after the 18-hour period toxicity effects were absent. From the finding in the peritoneal cavity of about 85% of the administered dose near the end of the period of toxicity it is apparent that some change must occur to permit improvement to take place in the presence of such large additional amounts of labeled antigen still available for absorption. In view of the fact

that much of the material is present as a solid phase at this time, concentration of the antigen in peritoneal fluids should remain fairly constant and it is likely that the change occurs in the reaction of the tissues of the rat to the antigen. Whether this takes place in the organs and other potentially susceptible tissues, or in the peritoneum itself, or in both is not discernible.

The fact that improvement takes place regularly over a period of less than three to six hours, and does so in the presence of large amounts of antigen available for absorption means (i) that the effects of this Flexner toxin are not cumulative in the rat over a period of a few hours, as contrasted with effects of tetanus toxin, for example, and (ii) that interaction with the tissues is either reversible or results in some chemical change by which toxicity is rapidly destroyed and the tissue elements involved are restored quickly. Since the effects of the toxin are not cumulative, degree of toxicity must be a function of concentration of toxin in extra- and intracellular fluids at any given time, with allowance for the unknown time constant, or lag, determined by the over-all rate of the interaction of toxin and target. The rate of this interaction process also would have bearing on the rate of recovery. The time of combination of target with enough toxin to produce symptoms of toxicity is no greater than a matter of minutes in view of the development of extreme toxicity in less than 15 to 20 minutes after intravenous administration of the toxin in succeeding experiments.

Of the total antigen found absorbed, 55% had been excreted in the urine, and 44% was found in the organs. Only 0.2% of the amount absorbed appeared in the feces eliminated during the experiment. Of the total activity found in the blood and the organs, muscle contained 27%, liver and spleen 34%, blood 10%, and all the rest 29%. It is worthy of note that of all the organs examined brain had no measurable activity. A feature of the findings was the rather general distribution of the toxin with no special predilection for the colon or other part of the intestinal tract. In fact, concentrations in the colon were especially low.

If the tissue concentrations observed were derived entirely from the blood contained in the tissue and if one may assume a mean blood content for the isolated, bled tissues of about 8% of their wet weight, or less, it is evident that tissue concentrations found to be in excess of 0.34 μg./g. (4.2×0.08) signify distribution of antigen in extravascular areas. This is seen to apply to all organs measured except the brain. The ubiquitous distribution in tissues indicates facile passage through vascular walls and is in keeping with the excretion of over one-half of the material found to be absorbed in 18 hours.

Assuming a mean extracellular and vascular volume of about 23% of wet tissue mass and a concentration of antigen in extracellular space no higher than that in blood, it is evident that tissue concentrations in excess of 0.97 μg./g. (4.2×0.23) signify that antigen entered the cells. This is true for all organs except brain, although muscle concentrations were only one-

third higher than this minimum. Except for muscle, colon, testis, and brain these cellular concentrations must have been of the same order as that of blood, or higher, namely, 4.2×10^{-3} μg./g. of blood or, approximately, of tissue. The dose given was approximately one-tenth of an $L.D._{50}$ dose for the rat on the basis of toxicity studies in mice and rats.[1] The $L.D._{50}$ level of antigen in rat blood, therefore, would appear to be about 4×10^{-2} μg./g. Deleterious effects on the unknown physiological target of dysentery antigen might be looked for in the concentration range of 1×10^{-3} to 5×10^{-2} μg./mg.

A comparison of the blood and urine activity levels shows that the material is concentrated by excretion through the kidney in the order of thirty-fold. During the 18 hours of the experiment the kidney cleared the blood of an amount of antigen 25% greater than that found in the blood and organs at the end of the period.

In the second experiment, designed to compare the little-known effect of intravenous introduction of the antigen with that of intraperitoneal injection, about 5% of the dose given to the first rat was injected into the femoral vein of another 100 g. rat which was killed four hours later. This animal looked toxic, with labored breathing, tenseness, ruffled hair, and unwillingness to move head or limbs on recovering from the ether, unlike a control rat given saline, and these symptoms continued for the four hours of the experiment. No urine or feces were passed. Blood level of antigen was 1.7 μg./g., which is of the same order as the level of 4.2 μg./g. in the first experiment when the antigen was administered intraperitoneally and the animal likewise killed during the period of toxicity.

The liver took up the antigen four times as avidly as the spleen. Concentration reached a peak in the lungs where it was twice as high as in the liver, the lungs being the first capillary bed reached by the material. Lower relative values for concentration in the lung in the first experiment may have been caused by filtration of the antigen in the process of absorption from the peritoneum (see also results of the third experiment).

The lower ileum was remarkable for having concentrated the antigen to an even greater extent than the spleen, but the colon remained low in antigen content which was again just a little higher than that in striated muscle. No labeled material was found in the brain.

This experiment confirms the ubiquitous distribution in extravascular space found in the first experiment, and, similarly, concentrations in organs in excess of 0.39 μg./g. (1.7×0.23) should signify the presence of antigen in the cells. Quite a number of organs, including colon, had levels of toxin too low for this specification.

In both this and the first experiment unequal distribution in various tissues and evidence for the presence of the antigen in the cells suggest that the reacting target exhibits specificity for the antigen and is unevenly distributed among the organs. Further, access of the antigen to the tissues

may be different for each organ. Access is determined largely by net permeability which suggests a degree of specificity also. The possibility that the distribution of the target may be caused by unequal distribution of some type of cell leads us to consider the types of cell distributed widely, such as

TABLE 3

INJECTION OF 0.25 MG. OF LABELED DYSENTERY ANTIGEN INTO THE ABDOMINAL AORTA OF A 100 G. RAT SACRIFICED AFTER THREE HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*Total antigen*	*100 × ratio of blood to tissue concentration*
	mg.	*Counts/min.*	*μg. antigen per g. tissue*	*μg.*	*Per cent*
Liver	134	96.5	12.0	76	5.0
Lungs	163	55.8	7.0	7	8.6
Spleen	156	36.0	4.2	4	14
Kidney	306	33.6	2.3	3	26
Duodenum	178	8.4	1.0	0.3	60
Blood	1000	18.6	0.6	3	100
Upper ileum	502	9.6	0.4	1	150
Stomach	163	3.6	0.4	0.5	150
Heart	101	1.8	0.3	0.2	200
Testis and epididymis	285	3.0	0.2	0.4	300
Lower ileum	363	2.4	0.1	0.3	600
Pancreas	1132	1.8	....	....	.....
Colon	250	1.2	....	....	.....
Adrenal	58	0	....	....	.....
Brain	200	0	....	....	.....
Leg muscle	364	0	....	....	.....
Total in organs				96	

lymphocytes, reticular types, etc. Presence of the target in the cells of Peyer's patches might explain for the various experiments reported in this paper the inconsistency as to relative uptake of antigen by given sections of the gastro-intestinal tract. However the lymphocyte is not such a cell since only 0.7 μg./g. were found in an excised lymph node.

The third experiment was carried out in the same way as the second except that the antigen was injected into the abdominal aorta through an abdominal incision. This was done to compare uptake by the lung with and without another capillary bed intervening between the site of injection and the lung.

The concentration of antigen as well as the total amount in the lung fell to about one-third of their values in the second experiment, although liver levels were about the same (Table 3). It is apparent that large amounts of antigen were filtered out by the muscle and other capillary beds through

which the antigen first passed. The absence of labeled antigen in other muscle (Table 3) shows that filtration was accomplished almost entirely on first contact. Apparently, much of the uptake by the lung in the second experiment was made up of larger particles filtered out in early contacts with the antigen, particles also filterable by muscle. The other findings confirm those of the first and second experiments.

TABLE 4

INJECTION OF 0.25 MG. OF LABELED DYSENTERY ANTIGEN INTO THE FEMORAL VEIN OF A 100 G. RAT SACRIFICED AFTER FOUR HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*Total antigen*	*100 × ratio of blood to tissue concentration*
	mg.	*Counts/min.*	*μg. antigen per g. tissue*	*μg.*	*Per cent*
Liver	320	965	30	199	4.7
Spleen	106	178	17	6	8.2
Lungs	122	128	9.0	8	16
Kidney	306	67.8	2.4	4	58
Lower ileum	267	32.4	1.5	3	93
Blood	1000	82.2	1.4	7	100
Duodenum	147	22.8	1.4	0.4	100
Stomach	178	23.4	1.2	2	120
Upper ileum	216	22.2	1.1	1	130
Middle ileum	332	29.4	0.9	0.3	160
Colon	343	22.8	0.7	1	200
Heart	176	7.2	0.6	0.3	230
Leg muscle	201	4.2	0.4	°16	350
Pancreas	700	14.4	0.3	0.2	470
Testis and epididymis	527	8.4	0.2	1	700
Thymus region*	164	2.4	0.1	<0.1	1400
Brain	442	3.6	0.1	0.1	1400
Adrenal	.....	4.8	....	.....	.....
Total in organs				249	
Urine	260	17.6	41.8	11	3.3

* Retrosternal tissue in region of thymus was dissected out.
° Total in all voluntary muscle, assuming weight of latter is equal to 40% of the weight of the whole animal.

The fourth experiment was carried out for two purposes: (i) to compare the distribution found in the preceding three experiments, in which a colloidal, semi-particulate antigen was used, with that resulting from injection of a completely soluble one, and (ii) to begin a series of three experiments to study the distribution of the antigen with respect to time.

For this purpose a purified antigen, B (see "Experimental"), was given in accordance with previous standard measurements of toxicity, the rat

showing the usual signs of toxicity in moderate degree during the four hours of life following injection into the femoral vein.

In general, the distribution confirms that already found, with low levels in colon, muscle, etc. (Table 4). The blood level was the same as that in the second experiment in which the antigen was given intravenously also. The comparable blood level can be taken as some measure of the degree of reproducibility of the experiments.

As expected, concentration in the lung fell, from 23 μg./g. in the second experiment to 9 μg./g. in the fourth, in keeping with the change in the collodial properties of the antigen. The difference in total uptake is 11 μg., which must have been available in this experiment for absorption or excretion elsewhere.

On the other hand, the liver unexpectedly took up 199 μg. of the antigen which was 80% of that injected. Also, the dose given to the animal was completely recovered in the organs. Had the liver taken up the same amount of the antigen, as it did in the second experiment, the total deposit in the organs would have amounted to 128 μg., a value very close to the total 135 μg. in the earlier experiment. Thus, about the same total amount of antigen was found in the organs other than the liver as was found when the more particulate antigen was used. It is evident that the increase in total uptake, yielding quantitative recovery of the antigen injected, was due to deposition in the liver. This occurred in competition with excretion by the kidney and suggests that the uptake was rapid, in keeping with the rapid onset of toxicity.

It is apparent from this finding and from any of the four tables that the affinity of the liver for the antigen is very much greater than that of any other tissue. Although it is possible that the reticulo-endothelial and related cells were responsible for all or most of the labeled material found in the liver, it is quite possible that they took up no more than a small fraction of the total. Comparison of uptake of the two antigen preparations by liver and spleen does not fit the usual rôle of the reticulo-endothelial cells and also in many of the tissues the vascular system shows little or no affinity for the antigen. Furthermore, calculation shows that the reticulo-endothelial cells would have had to concentrate the antigen in the order of two thousand-fold over the concentration in the blood. If the rôle of the reticulo-endothelial system were specific, the disease syndrome would appear to arise in some way from the relatively low concentrations in other organs since one would not expect the toxic effect on the animal to be mediated through the reticulo-endothelial system.

Therefore, it is quite possible that the antigen was present in high concentration in the hepatic cells. Also in the fourth experiment, as noted, the rate of uptake by the liver must have been rapid. It may be concluded that the biochemical target of this antigen appears to be present to a relatively large extent in the liver cells and that it combines rapidly with the

antigen. In fact, most of the target that is present in rat cells, probably more than 80%, seems to be contained in the liver.

The fifth and sixth experiments were designed to measure distribution after longer periods of time. Each rat was given the same material as in the fourth experiment, the fifth experiment being terminated at the end of 24 hours, the sixth at 72 hours. After 72 hours (Table 6), the liver, spleen, lungs, kidney, and blood lost about 60% of their antigen content at the four-hour point, namely, 151 μg. (Table 4). Of this, 49 μg. were taken up in muscle, testis, and epididymis, colon, thymus, and middle ileum, while the rest appeared in the urine. Several of the organs with increased uptake showed concentrations comparable to those in the liver. During the 72 hours, 145 μg. of antigen were lost in the urine in decreasing amounts each day. The feces showed negligible amounts.

The observations in Tables 4, 5, and 6, taken together, show that chiefly between the 24th- and 72d-hour observations, a large amount of antigen was removed from the liver and a few other organs and deposited in certain tissues or excreted. In the blood at 24 hours there was not more than 5% of the amount needed for the new deposits, and yet at 72 hours the blood retained levels comparable to those in the organs at that time. The removal of toxin from one tissue and its deposition in another shows that the toxin can move into the blood from cells in which it had previously been deposited from the blood. The deposition in certain other tissues of toxin liberated from the liver shows that the early process of uptake continues long after the animal has recovered. This process together with all other findings is characteristic of a dynamic equilibrium, which here seems to exist between antigen and target in the various cells of the body, mediated by the extracellular fluids and the blood. Apparently, the antigen interacts with the target in some reversible way. As previously concluded, this means that the toxic effect is dependent upon concentration of antigen at the site of the target and that the effect is essentially not cumulative. If the target were an enzyme system, one would look for inhibition of the enzyme by the antigen in competition with one or more substrates of that system.

Since some tissues showed an increase in concentration of antigen at 72 hours in the face of apparently falling blood levels but did not do so at the 24-hour point, it must be concluded that some change occurred in them by 72 hours signifying an increase in demand for the antigen. It seems possible that this may represent the earliest stages of a response, perhaps the immune response, on the part of some of the tissues to stimulus provided by the toxic action of the antigen.

Experiments

Synthesis of radio-p-iodoaniline. For the first experiment radio p-iodoaniline was prepared essentially by the methods of Michael and Norton[14] and of Chattaway and Constable.[8]

TABLE 5

INJECTION OF 0.25 MG. OF LABELED DYSENTERY ANTIGEN INTO FEMORAL VEIN OF A 100 G. RAT SACRIFICED AFTER 24 HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*100 × ratio of 24th- to 4th-hour concentration*	*100 × ratio of blood to tissue concentration*	*Total amounts of antigen*		
						Found in this experiment	*Lost or gained between 4th & 24th-hour*	*100 × ratio of 24th- to 4th-hour total*
	mg.	*Counts/min.*	*μg. of ant. per g. tissue*	*Per cent*	*Per cent*	*μg.*	*μg.*	*Per cent*
Liver	208	332	27	90	2.2	164	—35	82
Spleen	164	146	15	88	4.0	5	—1	83
Lung	146	33.0	3.8	42	16	4	—4	50
Kidney	183	20.4	1.9	79	32	3	—1	75
Middle ileum	216	10.8	0.9	100	67	0.2	—0.1	67
Duodenum	148	7.2	0.8	57	75	0.2	—0.2	50
Stomach	230	7.8	0.7	58	86	1	—1	50
Blood	1000	27.0	0.6	43	100	3	—4	43
Lower ileum	216	6.6	0.6	40	100	1	—2	33
Upper ileum	262	6.0	0.5	45	120	1	0	100
Pancreas	130	3.6	0.5	170	120	0.4	+0.2	200
Colon	230	6.6	0.5	71	120	0.3	−0.7	30
Thymus region*	232	4.8	0.4	400	150	0.2	+0.1	200
Testis and epididymis	474	4.8	0.3	150	200	1	0	100
Heart	138	1.8	0.3	50	200	0.1	—0.2	33
Lumbar muscle	574	4.8	0.2	50	300	°8	—8	50
Adrenal	....	1.8	....	...	.....	.....	.....	...
Brain	258	1.2	....	...	.....	.....	.....	...
Total in organs						192		

* Retrosternal tissue in region of thymus was dissected out.
° Total in all voluntary muscle, assuming weight of latter is equal to 40% of the weight of the whole animal.

TABLE 6

INJECTION OF 0.25 MG. OF LABELED DYSENTERY ANTIGEN INTO FEMORAL VEIN OF A 100 G. RAT SACRIFICED AFTER 72 HOURS

Tissue	*Wet weight of sample measured*	*Observed activity (corrected only for background)*	*Concentration in tissues*	*100 × ratio of 72d- to 24th-hour concentration*	*100 × ratio of blood to tissue concentration*	*Total amounts of antigen*		
						Found in this experiment	*Lost or gained between 24th & 72d hrs.*	*100 × ratio of 72d- to 24th-hour total*
	mg.	*Counts/min.*	*μg. of ant. per g. tissue*	*Per cent*	*Per cent*	*μg.*	*μg.*	*Per cent*
Liver	482	115	8.1	30	2.5	68	−96	41
Middle ileum	114	24.6	6.1	680	3.3	1	+1	500
Spleen	108	19.8	5.0	33	4.0	2	−3	40
Thymus region*	90	15.0	4.7	1200	4.3	1	+1	500
Testis and epididymis	152	17.4	4.4	1500	4.5	13	+12	1300
Colon	256	19.2	2.3	460	8.7	2	+2	670
Leg muscle	588	19.2	1.2	600	17	°50	+42	630
Lungs	112	3.0	0.8	21	25	1	−3	25
Kidney	204	4.2	0.7	37	29	1	−2	33
Cervical lymph gland	166	3.0	0.5	....	40	0.1	.....	....
Lower ileum	232	1.8	0.3	50	67	0.5	−0.5	50
Blood	500	3.0	0.2	33	100	1	−2	33
Heart	178	1.2	...	....	.....	.....	.....	....
Brain	340	1.2	...	....	.....	.....	.....	....
Duodenum	150	0.6	...	....	.....	.....	.....	....
Pancreas	130	0	...	....	.....	.....	.....	....
Adrenal	....	0	...	....	.....	.....	.....	....
Salivary gland	236	0	...	....	.....	.....	.....	....
Urine 1st Day	1000	284	7.2	....	.....	94	.....	....
Urine 2d Day	1000	87.0	2.2	....	.....	33	.....	....
Urine 3d Day	1000	26.4	0.8	....	.....	18	.....	....
Total in urine						145		
Total in organs						141		

* Retrosternal tissue in region of thymus was dissected out.
° Total in all voluntary muscle, assuming weight of latter is equal to 40% of the weight of the whole animal.

The synthesis was begun by adding to a mc. of carrier-free radioactive iodine in 1.6 ml. of 0.014 N NaOH solution 21.8 mg. of potassium iodide, 0.1 ml. of 2.25 N hydrochloric acid, and 5.76 mg. of potassium iodate. The free iodine was extracted by ether, the ether evaporated to dryness, and the iodine converted to iodine monochloride on a micro scale by the method of Chattaway and Constable.[6] Iodine loss in the form of iodine trichloride was minimized by providing conditions for early appearance of the latter as a precipitate, i.e., by passing chlorine very slowly into a chilled solution.

The radioiodoacetate was recrystallized once from 95% alcohol and the final product, radio p-iodoaniline, was recrystallized twice from a gasoline distillate collected between 75° and 135°, yield 2.31 mg., m.p. 60.5° to 61.2° uncorr. (reported m.p. 62°-63°, Brewster[5]). Similar results were obtained in preliminary syntheses using non-isotopic iodine.

For the subsequent experiments, radio-p-iodoaniline was prepared essentially by the method described by Brewster[5] and by Reiner and Keston.[20] This was done because the synthesis by way of iodine-monochloride required a month to complete even though each step had been carefully worked out beforehand; also in this simplified method the iodine is in volatile form for a shorter time. Since half of the iodine is converted to sodium iodide in the procedure, the alkaline filtrate from the reaction mixture may be treated with iodate and aniline again and the resulting alkaline filtrate retreated with iodate and aniline; the precipitates of p-iodoaniline then may be combined. The product was recrystallized as the sulfate from water once, and the p-iodoaniline was obtained by neutralizing with bicarbonate and centrifuging. In a preliminary synthesis the isolated iodoaniline gave a m.p. 62°-62.5°, uncorr. (reported m.p. 62°-63°, Brewster[5]). This synthesis required six days for completion.

Synthesis of azo compound of dysentery antigen and radio-p-iodoaniline. Two different preparations of the somatic antigen of Flexner dysentery bacilli, Type III, Z, were used, one referred to as antigen A, was purified extensively by very mild means.[2] Forming a milky solution with a fine particulate suspension, it was highly protective to mice on the basis of a standard level of toxicity[1] and was used for Experiments I, II, and III (see corresponding tables). The other, referred to as antigen B and purified by the method of Morgan and Partridge,[15] contained the carbohydrate and protein components of the antigen, dissolved completely giving a slightly opalescent solution and by mouse tests was found to be less protective per unit of toxicity than antigen A.

One and eight-tenths mg. of radio-p-iodoaniline were diazotized and coupled to 5 mg. of antigen, the coupling being carried out at a pH just alkaline to phenol-phthalein over a period of 15 minutes.

The light orange product was dialyzed against distilled water for 44 hours and the final dialyzed solution was raised to a volume of 4 ml., to which 36 mg. of sodium chloride were added. A portion of the antigen

solution was removed and found to contain a standardized radioactivity of 0.87×10^3 counts per second per mg. of antigen.

Counting procedures and preparation of samples. A sample of each organ was dried overnight at 95°, ground to a powder, resuspended in water or alcohol and dried as evenly as possible in a tin-plated steel cup at 95° for counting.

Results were corrected for absorption from a standard curve made by mixing various amounts of fresh liver with 10^{-3} rutherfords of iodine having 10 mg. of potassium iodide as carrier, and counting under identical conditions. Controls were run to test possible loss of iodine on drying. Separate absorption curves were used for blood and urine. The Geiger equipment was standardized for constant performance by a 60-cycle, 120 impulse/sec. input, by a C^{14} standard sample, and by use of an oscilloscope. Correction for decay was made from a decay curve for each preparation of I^{131}.

Injection of animals. The intravenous injections were made by making a small incision over the femoral vein in the inguinal region under light ether anesthesia and inserting a #26 needle into the exposed vessel. The arterial injection was made into the abdominal aorta after exposure through an abdominal incision.

Errors. Measurement errors of the counting technique were shown to be less than 5%, and frequent daily checks were made. Organ weights, wet, are accurate to less than 1%. Six different experiments attest to the validity of the data except for the 72-hour results which are based on one animal. Here the total balance of isotope fits the changes in distribution well and leaves only the possibility that this rat is abnormal.

Summary and Conclusions

When purified Flexner dysentery (Type III, Z) somatic antigen, labeled with I^{131}, is injected into the vascular system of a rat, it is distributed within four hours to most body tissues. Given intraperitoneally, only 10% is absorbed over 18 hours.

The antigen is present in tissues at 18 hours in such concentration as to indicate its presence within the cells of all organs except the brain. The colon is notable for its low concentration and liver for its extremely high one. Excretion occurs through the kidneys and only slightly in the feces, there being a thirty-fold concentration of the antigen by the kidney. The concentration value for the blood is close to that of many of the organs and the blood retains significant activity after 72 hours.

By 72 hours after injection much of the antigen has left the liver and several other organs and has been either excreted or taken up by muscle, sex organs, and a few other tissues.

It is concluded that this toxin-antigen after absorption passes freely through capillary and cellular membranes and circulates in body fluids in a dynamic and reversible equilibrium with a specific, cellular, biochemical target with which it interacts to produce in some way signs of toxicity in a manner of minutes after reaching the blood stream. The approximate $L.D._{50}$ concentration of antigen in rat blood appears to be about 40 μg./g. The target is contained to varying extent or specificity in the cells of most tissues, the liver being by far the most important and the colon not a primary site at all. In the liver the target may be contained chiefly in the hepatic cells. The other components of the equilibrium are excretion through the kidneys and absorption from some depot or source. Degree of toxicity is a function of concentration of the antigen in body fluids and the effect of toxin on the biochemical target is not appreciably cumulative.

After 18 hours there is evidence of a tissue reaction which lessens the effect of the toxin depot and by 72 hours there is evidence of tissue response in some cells, such as muscle, leading to an increased capacity for combining with the antigen. A rôle for this and other targets in the production of immunity by means of a local regenerative response is postulated.

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RESPONSE OF THE SPARROW TESTIS TO VARIOUS KINDS AND AMOUNTS OF GONADOTROPHINS*

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It has been known ever since the early investigations with gonadotrophins that the response of the bird to these substances is different from that of the mammal. In the bird, the testis is much more responsive to any type of stimulation than is the ovary, while in the mammal the reverse seems to be true. It was also shown relatively early that the chorionic gonadotrophins secreted in the urine of pregnant women brought about only slight response in birds,[5, 6, 10] while another chorionic gonadotrophin, which is found in the blood of the pregnant mare (PMS) but is not secreted in the urine, is one of the best gonadotrophic substances for stimulating the gonads of birds.[9]

There does not seem to be an adequate explanation of the above differences in response. The present experiments are an attempt to analyze the response of the sparrow testis to some of the gonadotrophins that have been used to stimulate this organ, with the aim of determining, if possible, the underlying principle responsible for the observed differences.

Materials and Methods

Most of the sparrows used in these experiments were trapped in the late summer and autumn but a few were obtained during the winter months. The birds were acclimatized to the laboratory before being used in the experiments, which were run during the non-breeding season. Almost all of the birds were juvenals at the time they were trapped. One hundred and eight male sparrows were used in these experiments. They were divided into four groups. The first group received PMS†; the second, whole sheep pituitary powder‡; the third, a partially purified pig pituitary powder¶; and the fourth, APL.§ Each of these groups was further divided to test the effects of large and small doses of hormone. The concentration of hormone was adjusted so that the required dosage was given in 0.05 cc. of solution. The number of animals receiving each treatment is given in Table 1.

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† P.M.S., the Pregnant Mare Serum preparation "Antex" was supplied by Ayerst, McKenna & Harrison through the courtesy of Dr. Edward C. Reifenstein, Jr.

‡ The whole sheep pituitary powder was an acetone dried preparation purchased from the Wilson Laboratories.

¶ The partially purified pig pituitary powder was obtained through the courtesy of Richard C. Bruner of Chappel Laboratories.

§ The "A.P.L." chorionic gonadotrophin was supplied by Ayerst, McKenna & Harrison through the courtesy of Dr. Edward C. Reifenstein, Jr.

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The birds were kept in wire cages 3x3x4 feet in dimension and located near a group of windows to allow as much light as possible. No attempt was made to control accurately the temperature of the animal room, which was heated during the winter, but which was subject to rather wide variations in temperature. The sparrows had access to food and water at all times. The food consisted of a dry mixture of equal parts of fine cracked chick grain and a commercial poultry growing mash.

The testes were usually fixed in Bouin's fluid and stained with Harris' hematoxylin and triosin, but a few were preserved in Zenker-formol and stained with Duplessis' modification of Mallory's trichrome stain[8] to determine whether there was any change in the connective tissue. All tissues were imbedded in paraffin and sectioned at 5 to 7 μ.

TABLE 1

RESPONSE OF THE SPARROW TESTIS TO VARIOUS KINDS AND AMOUNTS OF GONADOTROPHINS

No. of animals	*Hormone* treatment daily*	*Period of treatment days*	*Response†*			
			4 to 10 days	*10 to 20 days*	*20 to 30 days*	*Beyond 30 days*
30	25 i.u. P.M.S.	4-70	L.C.	L.C. + Sp.	Sp. + L.C.	Sp. + L.C.
10	12.5 i.u. P.M.S.	4-30	L.C.	L.C. + Sp.	Sp. + L.C.	.....
20	1 i.u. P.M.S.	7-33	Sp.?	Sp. + L.C.	Sp. + L.C.	Sp. + L.C.
10	2 mg. A.P.	27-36	.....	Sp.	Sp. + L.C.	Sp. + L.C.
8	10 r.u. P.P.	8-12	L.C.	L.C. + Sp.	.....	.....
10	1 r.u. P.P.	8-30	Sp. + L.C.	Sp. + L.C.	Sp. + L.C.	.....
10	100 i.u. A.P.L.	7-66	None	Sp. (4)	Sp. (8)	Sp. (8)
10	10 i.u. A.P.L.	7-30	None	Sp. (2)	Sp. (6)	.....

*P.M.S., pregnant mare serum; A.P., acetone dried sheep pituitary; P.P., partially purified pig pituitary; A.P.L., highly purified gonadotrophin from pregnancy urine.
† L.C., development of true Leydig cells; Sp., stimulation of spermatogenesis; ?, questionable response. The response was uniform within each group except the A.P.L. series, where the total number of animals that had responded by the end of each period is given in parenthesis. Androgen was produced in only 2 of the 10 sparrows that received 10 i.u. of A.P.L. and in only 4 of the 10 birds that received 100 i.u.

RESULTS

Effects of pregnant mare serum on the testes of sparrows. With doses of 25 i.u. of PMS daily the testes of sparrows produce androgen in amounts sufficient to stimulate the pigment forming cells in the bill by the fourth day. The early response in the testis is almost entirely a transformation of mesenchymal cells in the interstitial spaces into Leydig cells. The transformation occurs with such extreme rapidity that before 15 days it appears to be complete, and the entire intertubular space, except for the presence of capillaries and an occasional lymphocyte, is filled with Leydig cells (Fig. 1).

The growth of the tubules is much slower and is usually not appreciable before 7 days. Spermatogenesis proceeds at a rather uniform rate from this time on. It may be approaching completion by 15 days and continues as long as the hormone is given. As the testis enlarges, due to the tremendous

increase in tubular volume, the proportion of interstitial cells becomes less and less, although their number remains about constant, and they are found primarily in nests in the interstices between the tubules. There is apparently a slight increase in their number due to cell division, as an occasional mitotic figure is seen in the mature Leydig cells.

When PMS is used in daily doses of 12.5 i.u., the effect is essentially the same as with the higher dose except that the interstitial cell response does not proceed as rapidly. However, by 15 days the transformation of mesenchymal cells to Leydig cells is almost complete. There does not appear to be any difference in the rate of spermatogenesis in the two experiments.

The testicular response to a low dose (1 i.u.) of PMS is quite different from that brought about by large doses. The testis enlarges slowly, but by 7 days its diameter may have doubled. The only detectable response is early spermatogenic activity. By 15 days spermatogenesis has reached only the secondary spermatocyte stage. Spermatids may be present after 18 days (Fig. 2), and thereafter spermatogenesis gradually goes to completion. This development corresponds very closely to that which occurs during recrudescence in nature. An occasional cell identifiable as a Leydig cell may be seen between the tubules, but most of the cells are identical with those seen in the testis during normal seasonal development, which cannot be identified as Leydig cells. The bill begins to blacken in about a week and the color is added very much like that of birds in nature.

The response of the sparrow testis to whole sheep pituitary powder. The group of birds started on 1 mg. of acetone dried whole sheep pituitary powder showed no evidence of androgen production within 12 days. Therefore, the dose was raised to 2 mg. daily for the rest of the experiment. None of the birds was autopsied before 25 days from the start of the experiment because even with the larger dosage the bills do not begin to darken until about the fourth week of treatment. At this time the testes measure more than 4x4 mm. in the birds which show pigmentation in the bill. However, androgen is eventually produced by all of the testes if the pituitary powder is given for a sufficient period.

The development of the contents of the seminiferous tubules appears to be the same as that which occurs during the seasonal enlargement of the testis (Fig. 3). A few sperm are present in the tubules of some of the testes by 25 days but spermatogenesis is usually not complete until after 30 days of treatment. This slow development resembles very closely that which occurs in response to added light ration. The intertubular spaces differ from those in normal or light stimulated animals only in the fact that an occasional Leydig cell can be seen in some of the testes. The principal response to the acetone dried pituitary powder from sheep is stimulation of the germinal epithelium with only very slight stimulation of the Leydig cells.

The response of the sparrow testis to partially purified pig pituitary powder. When 1 r.u. of partially purified pig pituitary powder is given daily, the testes respond in a manner similar to that seen with the whole sheep pituitary powder except that spermatogenesis progresses at a more rapid rate, with the testes reaching in 12 to 15 days a stage comparable to that seen with the sheep pituitary powder after 25 days. With the pig pituitary powder spermatogenesis is complete by 25 days (Fig. 5). The bills begin to blacken slowly after 7 to 8 days, and isolated nests of Leydig cells can be found in the interstices between the tubules. As the tubules reach their maximum size these cells may still be seen packed between them. Their number does not appear to be any greater than at 8 to 12 days after the beginning of treatment, but they are always more numerous than following the administration of whole sheep pituitary powder. When the dose is raised to 10 r.u. daily, spermatogenesis does not proceed at any faster rate, but there is an increase in the formation of Leydig cells from the undifferentiated mesenchymal cells in the interstitial spaces. This process is not as consistent as in the experiments with the higher doses of PMS, but in one case the development of Leydig cells by 10 days was the most extreme seen by any method of stimulation (Fig. 4). In this case there was as yet no stimulation of the seminiferous epithelium. This bird was producing large amounts of androgen as indicated by the pigmentation of

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FIG. 1. Interstitial tissue development of the testes after 15 days of treatment with 25 i.u. of PMS daily. Note that stimulation is almost purely that of the interstitial elements. The tubules show only the earliest beginnings of development, while almost all of the cells in the intertubular spaces have been transformed into Leydig cells. x 220.

FIG. 2. Development of the sparrow testis after 18 days of treatment with 1 i.u. of PMS daily. Almost all of the development is in the seminiferous epithelium. There is only an occasional cell which can be identified as a Leydig cell in the narrow intertubular spaces. One Leydig cell may be seen in the upper left hand portion of the photograph. x 300.

FIG. 3. Development of the sparrow testis after the administration of 1 mg. of sheep pituitary powder daily for 12 days and then 2 mg. daily from the 12th to the 25th day. The testes resemble very much those found during normal seasonal recrudescence. x 300.

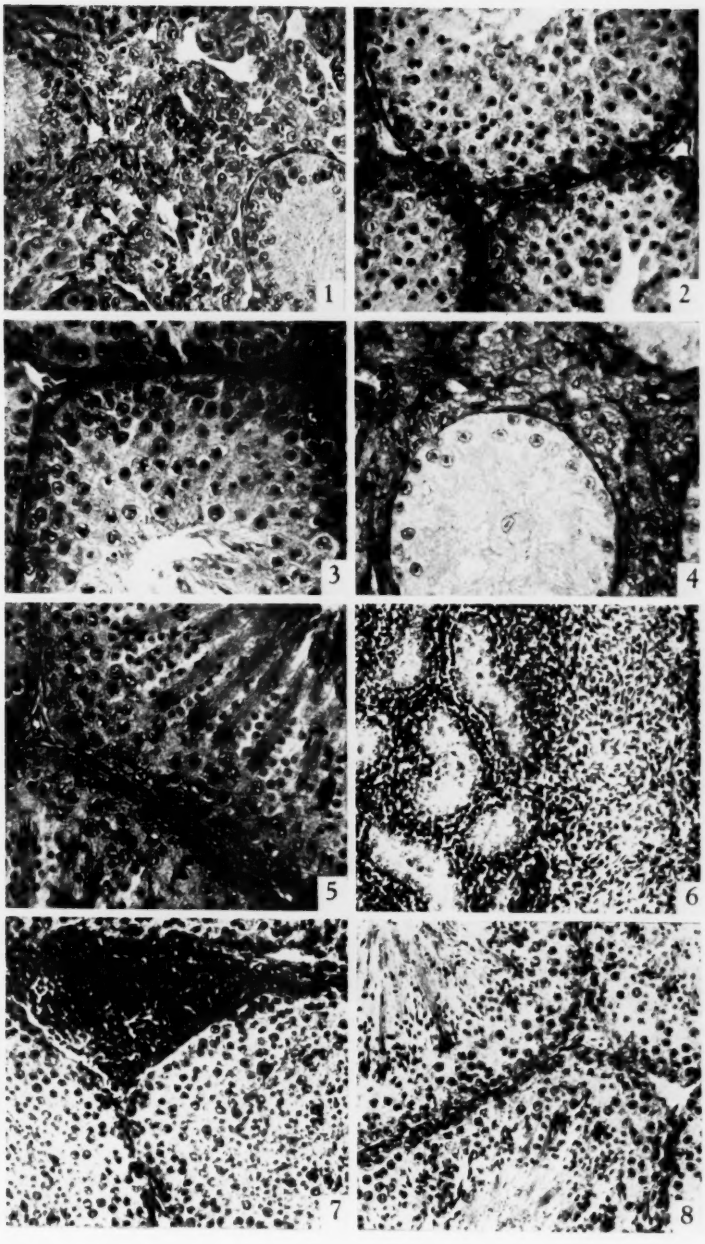
FIG. 4. Development of the Leydig cells in the intertubular spaces of the sparrow's testis following large doses of partially purified pig pituitary powder (10 r.u. daily for 10 days). Note the great hypertrophy and hyperplasia of the interstitial tissue without any change in the tubules except for a marked increase in the cytoplasm of the cells of the tubular epithelium. x 300.

FIG. 5. Complete spermatogenesis is seen after 25 days of treatment with 1 i.u. of partially purified pig pituitary powder daily. x 300.

FIG. 6. The testis of a sparrow which failed to respond after 66 days of treatment with 100 i.u. of APL daily. Note the extremely thickened connective tissue capsule of the testis, seen at the right. x 220.

FIG. 7. The response of the testes of one of the two birds which received 100 i.u. daily of APL and were producing androgen at the time of autopsy on the 15th day. Note the disorientation of the cells in the tubules and the large mass of lymphocytes in the intertubular space. x 220.

FIG. 8. Maximum stimulation of the testis with 100 i.u. daily of APL. This occurred after 30 days of treatment. x 220.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the bill, which was a deep slate blue in color. Except where there is appreciable stimulation of Leydig cells the testes reach almost as large a size as seen with the whole sheep pituitary powder before androgen is released in sufficient amount to cause pigmentation in the bill.

The response of the sparrow testis to chorionic gonadotrophin from pregnancy urine. Ten i.u. daily of APL gave very inconsistent results. Six of the 10 animals treated showed some stimulation of the testes by 28 days. The largest testis measured 6x5 mm. in the two greatest diameters, but spermatogenesis was not normal. The bills did not begin to blacken until about 21 days after the start of treatment and then in only two cases. Three more showed no blackening of the bill by 28 days even though the testes were enlarged up to 4 mm. in greatest diameter. Four animals showed no gross stimulation by 30 days after the start of treatment. In two of these the presence of a few sloughed cells in the lumina of the tubules indicated very slight abortive stimulation. Although spermatogenesis was abortive, a few mature sperm were seen in the largest testes. In all cases where there was stimulation there were numerous sloughed cells in the lumina, consisting primarily of the most mature stages that had been attained in the development of spermatogenesis. The interstitial tissue was not stimulated to form Leydig cells, but there was usually an increased number of lymphocytes in the intertubular spaces. There was also no production of connective tissue.

Increasing the dosage of APL to 100 i.u. daily gave only slightly better results than did the lower dose. Two of the birds had fairly dark bills by the end of the second week of treatment. Their testes measured 7x6 mm. in diameter on the 15th day. Two birds treated for 66 days showed no response (Fig. 6). Androgen was produced in some testes only slightly more than 3 mm. in diameter, but others attained a size of 7x5 mm. or more before secreting appreciable amounts of hormone. The testes giving the best response showed complete spermatogenesis, and the sperm bundles were characteristically arranged by the 30th day (Fig. 8). Spermatogenesis was never completely normal. There was always appreciable sloughing of cells of various stages of spermatogenesis even in the testes giving the best response. This sloughing was comparable to that seen in the case of the lower dosage. Leydig cells were lacking, but there seemed to be an increase in the number of mesenchymal cells in the intertubular spaces even in the testes with rapid development of the seminiferous tubules. There was a definite increase in the number of lymphocytes, which were often grouped in small nodules and sometimes formed a nodule of appreciable size (Fig. 7). In the testes which did not respond to the treatment, the seminiferous tubules appeared smaller than those of the quiescent testis and contained fewer cells. The mesenchymal cells in the intertubular spaces were more numerous and were also found in great numbers in the connective tissue capsule of the testis (Fig. 6).

In none of the experimental animals was there any increase in the amount of connective tissue in the intertubular spaces. Connective tissue stains showed only the thin connective tissue layer which forms the outer boundary of the seminiferous tubules. The response of the sparrow testis to the different kinds and amounts of gonadotrophins is summarized in Table 1. In Chart 1, the relative amounts of Leydig cell and seminiferous tubule stimulation are plotted in relation to the size of the testis when androgen production begins.

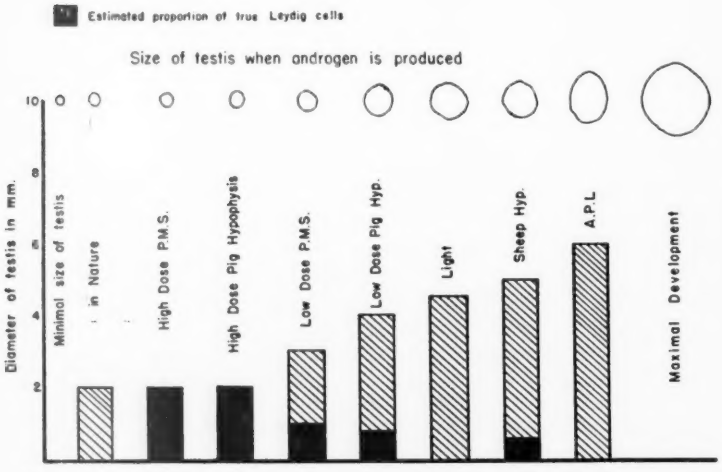

CHART 1. A diagramatic representation of the size of the testis with the proportion of tubular development to Leydig cell development found when androgen is produced under different conditions of stimulation. The response was uniform within each group where A.P.L. was given. Here, some animals failed to respond, while in those which did, the size of the testis when androgen production began varied from 3 x 3 mm. in both diameters to 7 x 5 mm. The latter figure is represented in the chart because it demonstrates the size which the testis can attain before androgen is produced when recognizable Leydig cells are not found.

DISCUSSION

The results obtained in the above study present further evidence that the sparrow testis differs from the mammalian testis in the way in which it responds to the various gonadotrophic hormones, as well as to different levels of the same hormone. It is evident, however, that in the sparrow, as in the mammal, there are two separate responses: (i) spermatogenesis and (ii) the development of interstitial cells of Leydig. It would appear that the first is induced by FSH and the second by LH. Witschi and Riley[11] state that "in the finches the FSH accelerates spermatogenesis and ovogenesis but does not stimulate the endocrine glands within the testis and ovaries."

As evidence for this they found that the injection of a purified FSH preparation (supplied by H. M. Evans) into the female sparrow was followed by follicular development without appreciable production of estrogen. There is no report of the effects of pure FSH or LH on the sparrow testis, but such preparations have been injected into hypophysectomized cockerels with the result that LH causes stimulation of interstitial tissue and androgen production, while FSH causes a stimulation of the seminiferous tubules without androgen production.[2] In the sparrow, Pfeiffer and Kirschbaum[4] were able to cause development of interstitial cells of Leydig and the production of androgen, without spermatogenesis, and they were able to stimulate spermatogenesis without the formation of any histologically recognizable interstitial cells of Leydig, although the latter testes produced androgen. These investigators did not use highly purified gonadotrophins. The present experiments add further evidence that there is a separate action of FSH and LH in the bird, but in order to prove this, it will be necessary to make careful histological studies after injection of the pure hormones.

Leydig cells as we understand them in mammalian testes have never been described in the testes of the normal or light-stimulated sparrow, either when male hormone is being produced or at any time during the normal cycle. However, Pellegrini[5] describes changes in the interstitial tissue of the sparrow's testis during recrudescence, and Sluitter and van Oordt[7] describe cytological changes in certain cells of the interstitial tissue of the chaffinch's testis which identifies them as cells which produce androgen but which cannot be recognized except by special cytological techniques. If a similar condition exists in the sparrow, it is possible that the differentiating mesenchymal cells may begin secreting androgens at a stage when they cannot be identified by the methods used for demonstrating Leydig cells in the mammalian testis, especially when they are being reoriented because of the enlargement of the seminiferous tubules. Under certain experimental conditions, however, these cells are stimulated to the extent that they are easily recognizable as true Leydig cells with routine staining procedures. There seems to be little doubt that the interstitial cells are derived from the primitive mesenchymal cells[4] which are so numerous in the intertubular spaces of the quiescent testis.

If we accept the hypothesis that FSH stimulates spermatogenesis without causing production of androgen and that LH stimulates the formation of interstitial cells of Leydig with production of androgen, a fairly logical explanation of the varied responses to the injected gonadotrophins can be given. The sparrow's testis is quite responsive to FSH, and the presence of LH appears to augment this reaction while at the same time stimulating the endocrine part of the testis. Nalbandov, Meyer, and McShan[3] have shown that there is a synergistic action of FSH and LH in the chicken. With low doses of both pituitary hormones and PMS the effect is mainly on spermatogenesis, since a very small amount of FSH is being synergized

by the presence of LH and therefore acts favorably on the tubules, yet the LH level is too low effectively to stimulate the mesenchymal cells in the intertubular spaces. When the doses are raised, the rate of spermatogenesis is not appreciably increased since the synergistic action of the LH on the FSH in the low dose had induced this process at about its maximum speed. On the other hand, the intertubular changes are greatly accelerated due to the increased amount of LH. With the very large doses the action of the LH is so increased that the development of interstitial cells may go to completion before the tubules are stimulated. The failure of PMS to induce spermatogenesis in the chaffinch testis[7] may very well be explained on this basis. It may be that with the large amount of LH the FSH acts as the synergist. In any event, under these conditions, the production of androgen as seen by the deposition of melanin in the bill is correlated with the production of true interstitial cells of Leydig. The size of the tubules or the stage of spermatogenesis is not closely tied to androgen production, as evidenced by the fact that when there is little change in the intertubular tissue, the testes reach a size of more than 4 mm. before androgen is produced.

The response to the chorionic gonadotrophin prepared from human pregnancy urine is very peculiar but can be explained if we assume that the LH of such a preparation is one that cannot be utilized by the bird. It is agreed by most workers that APL is primarily a luteinizing hormone and that only enough FSH is present to act synergistically with it. Therefore, it produces good responses in certain mammals where this LH is effective. In fact, in the mammal, APL is considered to be the most effective luteinizer available, not excluding the best pituitary sources, and is routinely used as a synergizer. It is very difficult to determine the amount of FSH in an APL preparation, but it is quite low. If the sparrow could use only the FSH available in the preparation there would be extremely low activity since there would be no synergistic action of LH. It may even be that the bird (sparrow in particular) can respond only to the pituitary FSH that is a contaminant in the preparation.

Since the sparrow responds very well to PMS, the chorionic gonadotrophin of the horse, which is not eliminated by the kidneys, the explanation cannot be that the sparrow responds only to pituitary gonadotrophins. Neither can the non-activity of APL be explained alone on the basis that it is a luteinizing hormone from a human source, for Witschi and Riley[11] have shown that, while the human hypophysis produces primarily an FSH response in the sparrow, LH is present in at least some of the human pituitaries (rat test) and is effective in causing the production of sex hormones in the sparrow, and gives the characteristic finch feather test for LH, which APL does not give.

The present work on the APL hormone prepared from the urine of pregnant women confirms the earlier work of Witschi and Keck[10] who obtained no response with 50 r.u. of a pregnancy urine extract, and a

response in only one animal with 20 r.u. of another extract. Since the birds used in the present experiment were near the beginning of the breeding season, it seems quite probable that only those birds whose testes had started to respond or were ready to respond to the normal factors which induce recrudescence of the gonads responded to the APL treatment. Therefore, Witschi and Keck were probably right in presuming that their single responsive bird might have already started to respond before the treatment. If the findings of de Fremery[1] do not fall into this category, they are difficult to explain. He apparently got much better results than any of the other workers using pregnancy urine extracts. He reported a good response (testis weight 100 mg.) after 13 days and complete spermatogenesis (testis weight 367 mg.) after 16 days with 100 i.u. daily of Pregnyl (a gonadotrophic preparation from pregnancy urine). The control testes weighed 3 to 4 mg. The sparrows were trapped in December and the experiment ran from January 17th to February 11th. The number of birds treated or the range of the response was not stated. The females did not respond.

There is ample evidence in the present experiment that the gonadotrophic hormone from pregnancy urine causes faulty development of the testis with accompanying atrophy of the accessory genital system as stated by Witschi.[9] Witschi, Stanley, and Riley[12] have also shown that the sparrow testis does not respond to a total of 800 r.u. of gonadotrophins present in the serum of a pregnant woman. The non-responsiveness to gonadotrophins from pregnancy urine has also been shown in doves, pigeons, ducks, and chicks.

There does not appear to be any reason for the great increase in lymphocytes in the testes treated with APL unless it might be that the primitive mesenchymal cells are abortively stimulated toward Leydig cells and then transform into lymphocytes as the regressing Leydig cells do (unpublished observations). It does not seem that the sloughing of cells within the tubules would call forth lymphocytes into the intertubular spaces.

Summary

One hundred and eight male sparrows were used to compare the response of the testis to various kinds and amounts of gonadotrophins. One i.u. daily of PMS induces the gradual formation of a few Leydig cells of secretory type and complete spermatogenesis. Higher doses of PMS, 12.5 to 25 i.u. daily stimulate the formation of massive numbers of Leydig cells from the mesenchymal cells in the intertubular spaces before spermatogenesis is initiated. Spermatogenesis proceeds only slightly faster than with the lower dose.

One r.u. of partially purified pig pituitary or 2 mg. of acetone dried whole sheep pituitary powder induce slight development of Leydig cells and gradual spermatogenesis. Spermatogenesis is somewhat more rapid with the partially purified pig pituitary, going to completion by three weeks as

against four weeks for the sheep pituitary powder. When the dose of pig pituitary is increased to 10 r.u., Leydig cell formation is greatly increased; sometimes all of the mesenchymal cells transform to Leydig cells without an increase in the response in the seminiferous tubules.

Ten i.u. daily of APL produces slight or no response in the seminiferous tubules and no interstitial response. When the dose is increased to 100 i.u. daily, there is still no development of Leydig cells and only a little better response in the germinal epithelium. Some animals show a slight enlargement of the testis by 18 days, and spermatogenesis may eventually go to completion in an occasional animal. In all cases there is evidence of abortive development, and some animals show no enlargement of the testis after 66 days of treatment. Lymphocytes are always numerous in the intertubular spaces even when there is no testicular enlargement.

Wherever Leydig cells are induced to form, androgen production is correlated with their presence.

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FURTHER STUDIES ON THE NUTRITION OF *HEMOPHILUS PISCIUM**

PHILIP J. GRIFFIN†

Hemophilus piscium is the etiological agent of "ulcer disease," an infection prevalent in certain salmonoid fishes.[19] Recent studies have indicated that diphosphothiamine (cocarboxylase) or adenosine triphosphate (ATP), but not hemin (X factor) and/or coenzyme I (V factor), could replace blood or other natural materials as supplements for the growth of this micro-organism.[8] The present report is concerned with the effect of a number of related compounds on the growth of *H. piscium* and with the development of a synthetic medium which supports the growth of this bacterium.

METHODS

Several strains of *H. piscium* were grown at 22° C. for 48 hours on nutrient agar supplemented with 10 per cent sterile defibrinated rabbit blood. A loopful of the resulting growth was emulsified in 10 ml. of the basal medium which contained proteose-peptone #3 (Difco) plus glucose[8] and incubated for 24 hours at 22° C.; visible growth was present at this time. One-tenth ml. of this culture was inoculated into 10 ml. of the fresh sterile basal medium and again incubated for 24 hours at 22° C. A faint growth response was evident at the end of this interval. The culture was then centrifuged at 2,000 r.p.m. for 10 minutes, the supernatant removed, and the organisms resuspended in 10 ml. of sterile basal medium. This process of sedimenting the organisms, removing the supernatant, and resuspending the bacteria was repeated twice. The organisms were finally suspended in 10 ml. of sterile basal medium and then diluted 1:5 with 0.9 per cent saline. One drop delivered from a 1 ml. pipette served as the inoculum for the various tests to be described. In this manner the transfer of accessory growth substances from the original blood-enriched medium was usually eliminated.

Materials to be tested for growth-promoting activity were sterilized by passage through Chamberland or Selas filters. Suitable controls were included to test the sterility of the added substances. After incubation for 96 hours at 22° C., growth was recorded in terms of optical density (Klett-Summerson photoelectric colorimeter; 660 mμ. filter).

RESULTS

Stimulatory effect of hemin. It has been shown that cocarboxylase or ATP are highly active in replacing such natural materials as blood or fish peptone in the growth of *H. piscium*.[8] However, the growth response to

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cocarboxylase or ATP approached, but never equaled, the growth which resulted in the basal medium containing either of these natural products. An absolute requirement for hemin had been ruled out in previous studies.[19] However, recent observations indicate that the addition of hemin to the basal medium containing either cocarboxylase or ATP produces a marked increase in the growth of *H. piscium*. The greatest stimulation occurred with 8-10 μg. of hemin per ml. of the basal medium. Frequently, the amount of growth was double that which was obtained with either cocarboxylase or ATP alone, and usually equaled the growth response to blood or fish peptone. Thus most, perhaps all, of the special activity of the natural products may be attributed to these substances.

Activity of thiamine and thiamine monophosphate. Since thiamine in concentrations as high as 100 mg. per cent did not support the growth of *H. piscium*, it appeared to be of interest to investigate the effect of thiamine in combination with either ATP or cocarboxylase. Cocarboxylase* was employed in a concentration of 10 μg. per ml. of basal medium, and varying concentrations of thiamine were added in a range of from 1 to 1000 μg. per ml. Similar tests were performed substituting ATP for cocarboxylase at a level of 50 μg. per ml. of basal medium. Although there were some slight differences encountered with the various strains of *H. piscium* employed, the data indicated that approximately 1000 μg. of thiamine per ml. of basal medium will inhibit practically all of the growth of *H. piscium* which normally occurred when 10 μg. per ml. of cocarboxylase were added to the basal medium. In the presence of ATP, inhibition of the growth of *H. piscium* was noted at slightly lower levels of thiamine. In other tests employing thiamine at a level of 1000 μg. per ml. of basal medium, the inhibition of growth could be overcome by progressively increasing the concentration of either cocarboxylase or ATP above 10 or 50 μg. per ml. respectively. Preliminary studies of the thiamine-cocarboxylase system suggest that the inhibition is competitive.

Thiamine monophosphate† in a concentration of 40 μg. per ml. of basal medium was tested for its ability to support the growth of *H. piscium*. This substance also supported the growth of the micro-organism and was found to be approximately 80 per cent as active as cocarboxylase. Pyridoxal phosphate,‡ employed at a concentration of 5 μg. per ml. of basal medium and glucose-1-phosphate, fructose-6-phosphate, and hexose diphosphate used in concentrations of 50 μg. per ml. of basal medium, respectively, proved to be devoid of growth-promoting activity for *H. piscium*.

Effect of adenylic acid and adenosine diphosphate. Adenylic acid¶ was

* Kindly supplied by Merck and Co., Rahway, New Jersey.
† Obtained from Dr. J. Melnick, Yale University.
‡ Provided by Dr. W. Umbreit, Merck Institute for Therapeutic Research, Rahway, New Jersey.
¶ Delta Chemical Works, New York; also Sigma Chemical Co., St. Louis, Missouri.

dissolved in M/50 KH_2PO_4, sterilized by filtration, and used in a concentration of 40 μg. per ml. of basal medium. The barium salt of adenosine diphosphate* (ADP) was converted to the sodium salt by dissolving the material in cold distilled water by the addition of 5N HCl, precipitating the barium as $BaSO_4$ with 1M Na_2SO_4, neutralizing as rapidly as possible with 5N NaOH, and filtering through No. 5 Whatman filter paper. The material was then diluted with distilled water, sterilized by filtration, and employed in the basal medium at a concentration of 40 μg. per ml. (calculated as the sodium salt). In the concentrations employed, these substances supported the growth of *H. piscium* but were not as active in this respect as 2 μg. of cocarboxylase per ml. of basal medium. If the optical density reading recorded for the growth response to 2 μg. of cocarboxylase per ml. of basal medium is arbitrarily assigned a value of 100 per cent, then the response of *H. piscium* to 40 μg. of adenylic acid per ml. of basal medium was approximately 15 per cent and the response to 40 μg. of ADP per ml. of basal medium was approximately 43 per cent. ATP in a concentration of 40 μg. per ml. of basal medium produced a response in the growth of *H. piscium* which was approximately 90 per cent of that occurring in the presence of 2 μg. of cocarboxylase per ml. of basal medium.

It should be noted that 1 to 2 μg. of cocarboxylase per ml. of basal medium were more active than any of the adenine compounds tested. An increase in the concentration of cocarboxylase above these levels did not result in any appreciable increase in growth.

Although slight strain differences apparently exist, an increase in the concentration of ATP or ADP above 20 and 40 μg. per ml. of basal medium, respectively, did not result in an increased growth response. The data concerning the growth of *H. piscium* in the presence of adenylic acid must be interpreted with caution since in about half of the tests no growth occurred, and in the remaining tests the growth response was weak.

Microbiological assay of adenylic acid, ADP and ATP for contamination with thiamine. In a previous report[6] it was suggested that the activity of ATP might be attributed to the presence of small amounts of cocarboxylase present as a contaminant. This possibility was tested by the application of a microbiological assay for thiamine to the various adenine compounds employed in this study. In the assay procedure, *Lactobacillus fermenti* served as the test organism.[18] Dr. H. P. Sarett of the School of Medicine, Tulane University, New Orleans, Louisiana, kindly performed the thiamine assays.

ATP was tested at levels of from 1.0 to 250 μg. per 10 ml. of medium per tube. The values obtained from several experiments in which 50 to 250 μg. of ATP were employed produced a growth response of *L. fermenti* which was duplicated with from 0.015 to 0.02 μg. of thiamine. The best repre-

* Sigma Chemical Co., St. Louis, Missouri.

sentative value appeared to be 0.018 μg. of thiamine per 100 μg. of ATP. ADP had very slight activity for *L. fermenti,* and the growth was not linearly proportional to the concentration of ADP. From 50 to 500 μg. of ADP per tube produced a growth response equivalent to that obtained with 0.002 to 0.005 μg. of thiamine. The assay of adenylic acid indicated that this material supplied essentially no thiamine when used at levels of from 50 to 500 μg. per 10 ml. of medium per tube. A few tubes occasionally gave slight turbidimetric readings, but these were not constant enough to permit any definite conclusion. The tubes showing growth were usually those with the intermediate amounts of adenylic acid and not those with the highest concentrations.

Adenylic acid, ADP, and ATP were tested in amounts of 10 and 50 μg. per tube in the presence of 0.01 and 0.03 μg. of thiamine to determine whether they stimulated the growth of the assay organism, *L. fermenti.* In all cases the growth with thiamine plus any of the adenylic compounds appeared to be additive, subject to the qualifications above.

The smallest concentration of ATP necessary to support the growth of *H. piscium* was 2.0 μg. per ml. of basal medium. If one assumed, on the basis of the thiamine assay of ATP with *L. fermenti,* that all of the thiamine was present in ATP as cocarboxylase, then the total amount of cocarboxylase present in 2 μg. per ml. of ATP necessary to initiate growth was approximately 0.0004 μg. per ml. Assays of cocarboxylase with *H. piscium* indicated that the amount required to initiate growth with the most sensitive strain (XI) was 0.003 μg. per ml. The difference in the required levels was also evident with the other test strains of *H. piscium* employed. Further assays utilizing the strain of *H. piscium* (XX) most sensitive to ADP have shown that 8.0 μg. of ADP or 0.05 μg. of cocarboxylase per ml. were necessary to initiate growth. On the basis of the thiamine assay of ADP with *L. fermenti,* if one assumes that 8.0 μg. of ADP per ml. of basal medium contained as much as 0.005 μg. of cocarboxylase, then it is apparent that this level also lies below the sensitivity of this strain of *H. piscium* to cocarboxylase. Therefore, it appears reasonable to conclude that the activity of ATP or ADP in supporting the growth of *H. piscium* is not due to contamination with cocarboxylase.

Growth of H. piscium in a synthetic medium. A semi-synthetic basal medium capable of supporting the growth of *H. piscium* was developed (Table 1). This medium contained several of the salts and vitamins required by many heterotrophic organisms which have been grown in defined media by other investigators. Good growth of strains XI and XIII of *H. piscium* occurred in this medium in 24 hours in the presence of added cocarboxylase. Strains XVIII and XX of *H. piscium,* however, required the addition of 0.05 ml. of an alkaline hydrolyzed yeast nucleic acid (Pfanstiehl). At least ten successive subcultures of all strains of *H. piscium* were made in this medium employing one loopful of the culture for each transfer.

Excellent growth also resulted when the above medium was further modified by employing a combination of 46 chemically defined compounds which included various amino acids, adenine, guanine, uracil, hemin, vitamins (including cocarboxylase), glucose, and mineral salts. All strains of *H. piscium* grew well and were transferred through at least 15 subcultures. The necessity of employing acid hydrolyzed casein and yeast nucleic acid was thus eliminated.[7]

TABLE 1

COMPOSITION OF A SEMI-SYNTHETIC MEDIUM FOR *H. piscium*

"Vitamin free" acid hydrolyzed casein	5.0 g.	Glucose	5.0 g.
L-cystine	0.1 g.	Ca. pantothenate	1.0 mg.
DL-Tryptophan	0.05 g.	Nicotinamide	0.5 mg.
DL-Serine	0.01 g.	Biotin	2.0 μg.
K_2HPO_4	0.25 g.	$MgSO_4 \cdot 7H_2O$	200 mg.
KH_2PO_4	0.25 g.	$MnSO_4$ (anhydrous)	1.0 mg.
NaCl	5.0 g.	$FeSO_4 \cdot 7H_2O$	20.0 mg.

Distilled water to 1000 ml.
pH 7.2 adjusted with 1N NaOH, then autoclaved at 121° C. for 10 minutes.
Cocarboxylase was added at a level of 2.0 μg. per ml. basal medium prior to inoculation.

DISCUSSION

The importance of supplementing a medium with either a prephosphorylated metabolite or a suitable transphosphorylating agent has been suggested by the work of many investigators,[12, 16, 20, 21] and strong evidence exists for the view that the phosphate derivatives of many of the vitamins, rather than the free forms, are active in metabolism. This applies clearly to thiamine in certain bacteria, for Lipmann[13] found that cocarboxylase rather than free thiamine mediated the oxidation of pyruvic acid. Barron and Lyman[1] reported that thiamine was inactive and cocarboxylase active in the oxidation of pyruvate by gonococci and hemolytic streptococci. Lohmann and Schuster[15] obtained similar data with yeast cells. It appears probable that thiamine is the precursor of cocarboxylase.[17] Some confirmation of this viewpoint comes from the demonstration of enzymes capable of phosphorylating thiamine which are present in a variety of cells. The available data indicate that adenosine triphosphate is probably the usual phosphorylating agent.[2, 14, 22]

The behavior of *H. piscium* may exemplify the profound enhancement in biological activity achieved by phosphorylating the vitamin moiety, since thiamine, in concentrations up to 100 mg. per cent did not support growth. In contrast with this finding, the phosphorylated derivative, diphosphothiamine, showed maximal activity in concentrations of 1 to 2 μg. per ml.

If the requirement for an exogenous supply of a growth factor reflects an impairment in synthesis, then the question arises as to the nature of the defect in the metabolism of *H. piscium*. The inactivity of thiamine and the activity of cocarboxylase may imply that the bacterium cannot convert thiamine to diphosphothiamine. However, thiamine monophosphate was highly active in promoting growth. Green and his associates[5] indicated that thiamine monophosphate could not replace the diphosphate in reactivating a carboxylase preparation, and Weil-Malherbe[22] reported that the monophosphate was inactive as the coenzyme of carboxylase from yeast. Therefore, if the diphosphate is the only compound possessing immediate cocarboxylase activity and if cocarboxylase is the compound of central importance in the metabolism of *H. piscium*, it may be reasonable to suggest that the response to the monophosphate is a reflection of its conversion to the diphosphate. It would then appear that the defect centers about the mechanism concerned with the initial phosphorylation of thiamine. The activity of the various adenine derivatives in a basal medium deficient only in diphosphothiamine poses the next question. If adenosine triphosphate is indeed the phosphorylating agent involved in the conversion of thiamine to diphosphothiamine, the activity of the adenine compounds in supporting the growth of *H. piscium* may be understandable. Lack of growth in an unenriched medium may be a result of the inability of the organism to synthesize certain substances, such as ATP, at a rate sufficient to satisfy the demands of the organism. The fact that adenylic acid would occasionally elicit a weak growth response might indicate that the organism still retains the ability to perform a limited amount of synthesis of ATP when given a suitable point of departure. This possibility parallels the suggestion that many highly specialized parasites are difficult to culture on artificial media due to their inability to synthesize an energy source such as ATP.[3, 11, 21]

Another suggestion may be advanced, based on the observation that thiamine will inhibit the growth of *H. piscium* in the presence of either diphosphothiamine or ATP. The organism may synthesize thiamine (or some other substance) so abundantly that a metabolic imbalance results. One might then assume that a high ratio of thiamine synthesis to cocarboxylase synthesis leads to a situation which is analogous to the *in vitro* demonstration of the inhibition of growth by an excess of thiamine. The addition of either an excess of a phosphorylating agent such as ATP, a suitable nucleus for synthesis such as adenylic acid or ADP, or a compound whose function may be directly interfered with in the competition, such as diphosphothiamine or thiamine monophosphate, may correct this imbalance.

With the notable exception of the work on *Hemophilus parainfluenzae* by Herbst[8, 9] and Herbst and Snell,[10] it appears that the growth requirements of various members of the genus *Hemophilus* have been investigated only in terms of the X and V factors. This is unfortunate and unnecessarily restrictive. Investigations such as those of Gingrich and Schlenk,[4] who have

demonstrated that the requirement for coenzyme I by so typical a member of the genus as *Hemophilus influenzae* is not absolute, suggest that nutritional studies of hemophilic bacteria be extended to include the utilization of various phosphorylated intermediates.

Summary

Although *H. piscium* lacks an absolute requirement for hemin, this compound in the presence of cocarboxylase or adenosine triphosphate produced a growth response which equaled that obtained with blood or fish peptone.

Adenosine diphosphate and thiamine monophosphate were found to support the growth of *H. piscium*. Equivocal growth occurred in the presence of adenylic acid.

The results of microbiological assays for thiamine in the various phosphorylated adenine compounds employed suggest that the activity of these compounds is not due to contamination with cocarboxylase.

Thiamine proved inhibitory to the growth of *H. piscium*. In separate tests it was found that this inhibition could be reversed by increasing the concentration of either cocarboxylase or ATP above those levels at which the inhibition occurred. Preliminary studies of the thiamine-cocarboxylase system indicate that the inhibition is probably competitive.

The organism was grown and subcultured repeatedly in a defined medium which contained various amino acids, adenine, guanine, uracil, glucose, hemin, several of the vitamins (including cocarboxylase), and mineral salts.

Acknowledgments

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Special thanks are extended to both Dr. S. F. Snieszko and Dr. S. H. Hutner for their many valuable suggestions, gifts of growth factors, and for their continued interest in following the work from its inception to the present.

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FRANCIS GILMAN BLAKE

1887-1952

On 1 February 1952, Dr. Francis Gilman Blake died of coronary occlusion in the Walter Reed Hospital in Washington, D. C. Dr. Blake was Chairman of the Department of Internal Medicine and Physician-in-chief of the New Haven Hospital and Dispensary from 1921 until the time of his death. He was Acting Dean of the Medical School from 1940 to 1941, and Dean from 1941 to 1947. On 1 January 1952 he was granted a leave of absence and became the Technical Director of Research, Medical Research and Development Board, Department of the Army, in Washington, D. C. In addition to his many curricular and extracurricular interests and responsibilities, he was President of the Corporators and a Consulting Editor of the *Yale Journal of Biology and Medicine*. In accordance with plans formulated many months ago, the June 1952 issue of this *Journal* will be dedicated to Dr. Blake.

EVENTS

SOCIETY OF SIGMA XI

X-RAY STUDIES OF MATERIALS OF BIOLOGICAL INTEREST. By Dr I. Fankuchen, Professor of Applied Physics, Polytechnic Institute of Brooklyn.

The science of X-ray crystallography commenced in 1911 in Munich, where a group of physicists headed by von Laue applied scattering theory to the diffraction of X-rays by the single atoms in regularly arranged molecules. It was found that substances of varying degrees of molecular organization gave different sorts of diffraction patterns on a photographic plate. Amorphous rubber on one hand gives hazy concentric halos, while single pure crystals give patterns of discrete spots. From appropriate measurements of the location and intensity of these spots, it is possible to determine the dimensions of the unit cells in crystalline materials. By Fourier analysis one may locate accurately the individual atoms in at least the smaller molecules and draw some conclusions about the nature of the interatomic bonds. X-ray analysis gives relatively accurate values for the molecular weights with micro-quantities of materials. A special small-angle scattering instrument has been devised to give data on the intermolecular arrangement, under various conditions of hydration, of such huge molecules as the tobacco mosaic virus, each of which is a crystal in its own right.

W. P. McN.

PLANT SCIENCE SEMINAR

February 8, 1952

STUDIES ON *LABYRINTHULA*. By Stanley M. Watson, of the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

The slime mold *Labyrinthula* is interesting, like all slime molds, because it is a "naked mass of protoplasm"—a grouping of amoeboid cells acting in concert. This genus was thought to be associated with the "wasting disease" of the marine eelgrass *Zostera*, once an important member of the shallow water community of the eastern coast of North America, but now virtually absent. It was not possible to maintain *Labyrinthula* in pure culture at the time the experiments on pathogenicity were being made, so that the conditions of Koch's postulates could not be fulfilled.

It has recently been found that *Labyrinthula* can be grown on either blood or serum agar. This fact has not yet been used in the study of the "wasting disease." It is remarkable that in recent years *Labyrinthula* has been found not only on diseased *Zostera* plants, but also in such healthy stands as remain on the Atlantic Coast. In addition, *Labyrinthula* has been found wherever looked for on the Pacific Coast, where the "wasting disease" is unknown. All this, of course, is only circumstantial evidence that *Labyrinthula* is not the cause of the disease in at least some strains of *Zostera*.

There is a keen interest in this organism now because of its convenience as a tool for the study of the properties of protoplasm. The property now being investigated is locomotion. The individual cells extend strands of protein along the surface of the substrate; these strands branch and grow and fuse with the strands of other cells when they chance to meet. Finally the mesh-like structure characteristic of the genus is formed. The cells stream

trolley-wise along the mesh, always away from the center of the colony. A gradient in the concentration of nutrients may be responsible for the direction of the motion. The hypothesis that the properties of actinomyosin are responsible for the motion itself is being investigated.

The formation of the mesh and the motion were shown in a film.

R. R. L. G.

YALE MEDICAL SOCIETY

February 13, 1952

MULTIPLICATION OF POLIOMYELITIS VIRUSES IN TISSUE CULTURE. By Nada Ledinko, John Riordan, and J. L. Melnick.

The elusive poliomyelitis virus has been pursuaded at last to propagate *in vitro* in non-neural tissue. The roller tube technique, employing monkey testicular tissue, provides conditions for fibroblastic proliferation in which the virus may subsequently betray its presence. In contrast to normal cultures, tubes containing virus demonstrate fibroblastic degeneration with shrinkage and granulation. Controlled testing of the method substantiates its validity for readings of virus titrations and serum neutralization endpoints taken at four days following inoculation. At this time, non-specific tissue degeneration has not yet begun to take place, nor does "breakthrough" of the virus in the presence of homologous serum occur. The endpoints thus accumulated concur with the previous neutralization endpoints in mice and monkeys, giving impetus to the belief that *in vitro* quantitative and qualitative antibody determinations as well as virus titrations are feasible. There is a linear, logarithmic relationship between amounts of serum and virus used in a tissue culture neutralization test.

Metabolic and growth studies undertaken with this method indicate further spheres of its use in the future. It is possible, for example, to show a correlation between the amount of fibroblastic degeneration and inhibition of metabolic activity of infected tissue culture. Furthermore, on the basis of a growth curve determined in monkey testicular tissue, the virus may be passed for many generations. The Lansing strain of this laboratory shows itself on passage to decrease its usual pathogenicity for mice while retaining the capacity to infect monkeys. Thus, modification for one host may occur without affecting pathogenicity for the host in whose tissues the virus is grown. Such modifications will play a prominent part in the future consideration of tissue culture virus for the rôle of vaccine.

O. L. K.

REGENERATION OF THE AMPHIBIAN LENS. By L. S. Stone.

Removal of the lens of the adult *Triturus viridescens* results in complete regeneration of a new lens from the dorsal pupillary margin of the iris in 25-30 days. Histological sections of the iris show that the new lens is regenerated from the pigmented epithelial cells of this region. Thus the regenerated lens arises from epithelial cells which were formerly derived from brain ectoderm, while the original lens arises from embryonic surface ectoderm.

After removal of the lens the pigmented cells at 12 o'clock on the free pupillary margin of the iris begin to swell, lose pigment and undergo mitosis to give rise to a clear bud or vesicle which eventually detaches itself from the iris and forms the new lens. The ability to regenerate a lens is widely distributed over the dorsal half of the iris and is not confined to the

free pupillary margin. This is shown by the experimental production of secondary lenses after insertion of pieces of pliofilm or cornea into the dorsal iris. If non-lens-producing ventral iris is grafted so as to replace a strip of dorsal iris, two separate lenses are produced by the remaining separated portions of the dorsal iris.

The regeneration of a lens can be inhibited by: (1) the presence of a transplanted normal lens of the same or another species; (2) the presence of a thirty-day or older lens regenerate; (3) the injection of aqueous humor from eyes containing lenses; (4) the presence of some carcinogens. Implantation of a plastic lens does not produce any measurable inhibition of lens regeneration.

Observations were also made on the regeneration of neural retina from underlying pigment cells. All of these experiments have a particular bearing on the question of the potentiality of specialized cells to dedifferentiate and give rise to new specialized cells of another type. These experiments are the most clear-cut proof that specialized cells can change their form and function, undergo proliferation, and give rise to new cells of an entirely different type. Since all the changes in these pigment cells can be followed in detail, they become a unique tool for studying fundamental principles governing regeneration.

The presentation of the paper was accompanied by the showing of a film summarizing the results of the study of lens regeneration.

J. M. Q.

Some Technical Considerations in the Surgical Treatment of Congenital Malformations of the Heart and Great Vessels. By William W. L. Glenn.

A survey of 102 patients operated upon in the last three years was presented. The mortality in this group was 4.9%.

Fifty cases of patent ductus arteriosus were treated surgically. Division of the ductus was found preferable to ligation alone. A modified Potts procedure was employed, the vessels being fixed by a continuous suture before division, to prevent slippage of the clamp. Twenty cases of the tetralogy of Fallot were treated by the Blalock-Taussig operation. Two facts of general consequence were learned from these procedures: (i) Cardiac massage often has to be maintained for long periods of time in treating standstill, to prevent its recurrence; (ii) the usage of intravenous procaine is not warranted, because it creates more of a hazard by interfering with the restarting of the heart from standstill, than the risk of fibrillation, which it prevents. A modification of the Blalock clamp was also introduced which avoids vessel-injury. Fifteen coarctations of the aorta were operated upon by resection and end-to-end anastomosis. The possibility of anastomosing the left subclavian artery to the distal aorta and the use of aortic grafts was considered. Direct X-ray examination is used to establish the presence of calcifications, which may seriously impair the solidity of the sutures. For complete transposition of the great vessels the creation of septal defects or re-transposition by shunts may be considered. To establish the correct approach to double aortas catheterization or retrograde angiography are used. Pulmonary stenosis was treated with Brock's valvulotomy, yielding satisfactory results. In the future, open-heart surgery may make possible repair of mitral atresia, common truncus, and anomalies of the great veins.

N. A. H.

MICROBIOLOGY SEMINAR

February 14, 1952

THE FORMATION OF BETA-GALACTOSIDASE. By Dr. Gabriel Lester, Chas. Pfizer Company, Brooklyn, New York.

To study the formation of adaptive enzymes it is desirable to have a method better than the standard ones of measuring growth, manometric changes, or the decomposition of a substrate by whole cells. Such a method is available in the case of the Beta-galactosidase (lactase) of E. coli K-12. This is a classical enzyme system, stable and easy to obtain. The cell extract used was made in the usual manner, and contained 70-80 per cent of the cell nitrogen. The assay for the presence of the enzyme was made using the synthetic galactoside consisting of galactose joined with a *beta* linkage to ortho-N-phenol. This is colorless in solution, but yellow in basic solution when the *beta* linkage is broken. As little as five millimicromols can be detected colorimetrically.

The influence of various sugars upon the formation of lactase was studied by this method. It was found that certain sugars greatly stimulated lactase production, notably melibiose, which was more effective than lactose itself. In all instances the identity of the enzyme produced was checked serologically and by physical methods. It had been thought that perhaps melibiase and lactase were the same enzyme, but this was not so.

It might be expected that if the stimulation of lactase formation were due to structural similarities between the various sugars, an inverse relation might exist between the effectiveness of the sugars as stimulators of lactase production and as inhibitors of lactase activity. No such correlation could be found. Other hypotheses have not yet been checked.

R. R. L. G.

PHARMACOLOGY SEMINAR

February 14, 1952

STUDIES ON BARBITURATES. By Bernard B. Brodie, Chief, Section on Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

Thiopental is an ultra-short acting barbiturate if given in a single small dose while successive injections will produce anesthesia for long periods. This paradox stimulated investigations of the distribution and metabolism of the drug and some of its analogues in both man and dog. Such studies demonstrated that 80 per cent of administered thiopental is localized in fat depots. An equilibrium exists between plasma levels of the drug and its concentration in fat, the plasma levels diminishing as the concentration in fat increases. The lipophilic nature of the drug helps to explain its dual property of short action with small doses and long action with successive doses: anesthesia is curtailed by removal of the drug from plasma, but when successive doses are administered, the fat becomes saturated, the drug is slowly released to the plasma, and anesthesia ensues for long periods. The animal body, in this regard, approximates a biphasic water-fat system. And like its test tube counterpart, the distribution of drug between phases is influenced by pH. A fall in plasma pH, induced by breathing carbon dioxide through a Douglas bag, was accompanied by a fall in plasma levels and an increase in fat deposition of thiopental. Other ultra-short acting barbiturates such as evipal are similarly localized in fat, but fat depots do not markedly accumulate the long-acting pentobarbital.

Not only are thiopental and its oxygen analogue, pentobarbital, localized differently, but their metabolisms probably proceed through different enzyme systems. The metabolite of pentobarbital has an hydroxyl group on the side chain, that of thiopental a carboxyl group. The conversion of a methyl group in thiopental to a carboxyl group probably involves a series of reactions which occur too rapidly for the intermediate metabolites to be isolated. The postulated sequence of reactions for the conversion of the alkyl group to a carboxylic acid is a dehydrogenation followed by hydration to form an alcohol; oxidation of the alcohol yields an aldehyde which, after hydration and dehydrogenation, forms the acid. The final metabolites of thiopental and pentobarbital are not hypnotic.

Although the localization and metabolism of a drug are determinants in its pharmacological activity, the phenomenon of tissue adaptation is an important consideration. Thus, the central nervous system demonstrates a resistance to thiopental which can be regarded as an acute, non-persistent form of tolerance. A patient may awake from thiopental anesthesia with no change in the plasma levels of the drug. This type of tolerance is desirable, for it minimizes the duration of post-anesthetic narcosis and somnolence.

Since there is striking species variation in response to barbiturates and other drugs, caution must be exercised in predicting the response of humans to a pharmacological agent on information obtained from experimental animals. In the dog, for example, pentobarbital is a shorter-acting drug than pentothal. The reverse holds true for man.

J. P. G.

PHARMACOLOGY SEMINAR

February 21, 1952

PHARMACOLOGY OF HYALURONIDASE. By Joseph Seifter, Wyeth Institute for Applied Biochemistry, Philadelphia, Pennsylvania.

The clinical usefulness of the extracellular enzyme, hyaluronidase, rests on its ability to degrade interstitial ground substance. In hypodermoclyses it increases the rate of absorption three-fold with the avoidance of post-clysis edema. It doubles the area anesthetized by a procaine-epinephrine mixture. Recently it was observed that this enzyme alters the characteristics of urine. Within one-half hour of its subcutaneous injection, crystals covered by a protective colloid are observed in the urine; after one hour, the urine is again clear. Hyaluronic acid, a powerful dispersing agent, converted pathologic crystalluria to normal *in vitro,* an observation which explains the *in vivo* effect of hyaluronidase upon crystalline substances in the urine. Physiologically, hyaluronic acid may act as a peptizing agent to prevent precipitation of substances in the extracellular spaces.

Hyaluronidase has been found only in mammalian testes, but is produced by some bacteria, especially streptococci. The available preparation is an extract of the testes of cattle. Despite its impurity—it is only 20 per cent pure—and contamination with traces of histamine, hyaluronidase is one of the safest therapeutic agents available. No toxic effect has ever been reported. Its LD_{50} is about 10 g./kg. given intravenously, its margin of safety at least 100,000. It is without demonstrable effect on isolated organs, capillary permeability, urinary volume, or hematocrit. After one hour, a massive dose of the substance is no longer detectable in the blood.

J. P. G.

NEUROLOGICAL STUDY UNIT

March 11, 1952

TRANSORBITAL LOBOTOMY—MINOR SURGERY FOR MAJOR COMPLAINTS. By Walter Freeman, Professor of Neurology, Georgetown University, Washington, D. C.

Division of the thalamofrontal projections by the transorbital route combined with a deep inferior cut has been employed since 1948. Using electroshock for anesthesia the skull is entered through the conjunctival sac and the superior orbital plate. Thus the brain is approached through a "naturally sterile" area, and all major blood vessels are avoided, greatly decreasing postoperative complications. The leukotome then is advanced into the white matter, making lateral, medial, and downward cuts, the latter dividing the highly important fibers to the median ventral quadrant of the frontal lobe. Withdrawal of the instrument completes the procedure. This technical discussion was supplemented by a motion picture, another one presenting the clinical study of a catatonic patient treated by this method.

The relative simplicity of the procedure makes it the treatment of choice in hospitals where large numbers of patients have to be cared for by a small staff. In cases of terminal malignancy where relief of pain is essential but major surgery such as the conventional lobotomy would not be tolerated, the transorbital method is applicable and yields satisfactory results. In the treatment of middle-life psychoses, especially in their early stages, this technique seems to be superior to the others. However, in advanced schizophrenics and children with longstanding psychoses major lobotomy gives the best results.

N. A. H.

BOOK REVIEWS

THE KIDNEY, STRUCTURE AND FUNCTION IN HEALTH AND DISEASE. By Homer W. Smith. New York, Oxford University Press, 1951. 1072 pp. $15.00.

The appearance of a treatise on the kidney by Homer Smith is an event of the first importance to physiologists and physicians alike, since no one in this era has contributed more brilliantly than Smith to basic knowledge of the functions of the kidney. The wealth of material in the volume can be estimated from the facts that it contains 889 pages of text, 153 pictures, 3 plates and a bibliography of 2,300 references. As a compendium and reference book alone the volume is indispensable.

It is, as the title implies, concerned with the kidney, almost to the exclusion of the animal which this organ serves. This is both surprising and disappointing from Smith, who has made such fascinating contributions to the comparative physiology of the kidney and its adaptation to evolutionary and environmental vicissitudes. The present volume opens with a short chapter on the anatomy of the kidney. Consideration of comparative physiology is postponed to page 520, although experimental data from all species are cited earlier. The treatment of the subject is almost purely categorical. Lacking also throughout the book are the philosophical attitude and the sprightly style that have lent so much charm to everything that Smith has written, not only *Kamongo,* once a Book-of-the-Month Club selection, and that gem, *Plato and Clementine,* but also his *Physiology of the Kidney,* and his special scientific articles. Despite the 2,300 references, this is essentially an autobiography or record of the accomplishments of the Smith school with the aid of clearance techniques. In fact, after a cursory consideration of the classical work of Richards and his associates, the reader encounters 200 pages devoted to a detailed description of the principle of renal clearances, techniques for their measurement, and the clearances of a great variety of substances. This is the thesis of the volume. The use of the clearance technique is made the criterion of validity. In fact, the field is still further narrowed by the establishment of special ground rules. For example, on page 61 studies on falling plasma curves are disparaged. While the difficulties of measuring clearances under these conditions must be recognized, the possibility cannot be denied that the reactions of the kidney to rising and falling concentrations of solutes may not be identical.

This kidney of Smith's seems to have a fence about it with a *No Trespassing* sign on it. In discussions of Wolf's studies, these surprising statements appear: "The analysis of renal function must necessarily progress slowly, but it seems doubtful to the writer that it will be accelerated by the empirical approach" (page 343), and "in the absence of data on plasma concentration these data are emenable only to empiric description" (page 351). Wolf's exquisite demonstrations of biological autonomy cannot be dismissed in this arrogant manner. Wolf is interested in the kidney as an agent in the maintenance of this autonomy. His experiments are as precisely quantitative as Smith's; but his methods are different and he recognizes the value of inference. His work on the regulation of thirst and renal function by the effective osmotic pressure of the body fluids demonstrates that the body responds to variations that are beyond the sensitivity of present methods of measurement, but not beyond the capacity of human

ingenuity and inference, qualities which Smith has so strongly displayed.

Evidence in the voluminous bibliography is not cited without prejudice. This is peculiarly flagrant in the discussion of osmotic diuresis. On page 321, in disposing of Seldin and Tarail, he states: "Their argument, however, is based in part upon the assertion that, during osmotic diuresis caused by sodium sulfate and urea, chloride excretion is not increased, which is contrary to the evidence cited above." The evidence cited appears to be an undocumented statement of opinion about urea in a footnote on page 310 and articles by Schou on excretion of sulfate. Schou studied the rabbit, an animal about which Smith expresses his suspicions later in the volume. The other authors in his bibliography who have examined the excretion of sulfate in dog and man are unanimous in their conclusion that it suppresses the excretion of chloride; but in this connection they are not mentioned. Similarly, in the discussion of the tension of CO_2 in the urine, Mainzer and Conway are not even cited.

There are inexplicable omissions and what must be thoughtless errors that give the impression that the work was compiled in haste. In the discussion of acid-base equilibrium, Chapter XIII, for example, the reciprocal relation between bicarbonate and chloride excretion is recognized; but the significant facts that breathing an atmosphere enriched with CO_2 promptly elicits the excretion of ammonia and that overventilation promotes reabsorption of Cl^- are not mentioned. On page 376 ammonium sulfate is listed as an acidifying salt, although in another connection it is remarked that the sulfate ion is absorbed with difficulty from the gastro-intestinal tract.

To the reviewer the chapters seem to have been arranged with no logical sequence. The index is quite inadequate. Fortunately, it is supplemented by the expedient of indicating in the bibliography the pages on which each reference is cited.

Withal the volume stands as a great contribution and an indispensable reference book. Its defects would weigh less had we not learned to expect so much more from Homer Smith.

JOHN P. PETERS

PATTERNS OF DISEASE. By Frank L. Apperly. Philadelphia, J. B. Lippincott Co., 1951. 456 pp. $8.00.

Undoubtedly there is room for a good text on physiological pathology—especially one as up to date as Apperly's. It is disappointing, therefore, to find that the author has seriously underestimated the student and "young doctor in practice" for whom the book was intended. He includes some excellent summaries of the physiology of the kidney and liver as well as some helpful clinical notes. The discussion of fever, for example, might be of interest to the preclinical student. But these virtues would seem to be offset by a number of deficiencies. To mention a few which bothered this reviewer: there is no bibliography; the diagrams are lifeless and often seem rather superfluous; the index is incomplete, making it a poor reference book; much of pathology is completely left out—notably the skin. In speaking of omissions, one must in fairness to the author credit him with having made a very small book out of a very large subject. He is clear and economical, if somewhat given to parentheses and lists instead of sentences. In such a book there is no room for experimental evidence and the reader is left ignorant of the great arguments of pathology. Some students and medical educators feel that such a presentation is adequate, in fact prefer-

able, but there are others who view the educated man as one who can proceed from the observed facts to his own conclusions. Since the author assumes a thorough knowledge of the preclinical sciences, including anatomical pathology in his reader, one wonders if this reader might not be ready for the more advanced books on function.

The printer has come to the aid of the student with an easy-to-read type face, but the paper and cover are too flimsy to withstand much heavy studying.

O. L. K.

METHODS IN MEDICAL RESEARCH. M. B. Visscher, Editor-in-Chief. Chicago, The Yearbook Publishers, Inc., 1951. 306 pp., illus.

This volume includes sections on histochemistry, determination of fluid and electrolyte distribution, procedures used in gastro-intestinal studies and tissue culture. A brief résumé of the topics presented is as follows: 1. Histochemistry by G. Gomori: methods of detection of inorganic and organic substances including enzyme activity with a section on buffers. 2. Fluid and electrolyte distribution, edited by L. B. Flexner: measurement of total body water and plasma volume by different methods; calcium ion concentration and flame photometry. 3. Studies on gastro-intestinal pressures, innervation, and secretions edited by J. P. Quigley: methods of determining intraluminal pressures of the gastro-intestinal tract and secretory activity of pancreas and stomach. 4. Tissue culture methods, edited by L. M. Pomerat: preparation of media for specific purposes; methods for quantitative studies on cells *in vitro* (including cell growth) and for studies upon organized growth as of tissue; methods of observing the action upon cells of physical and chemical agents including radiation as well as drugs in assay or screening tests; brief presentations on phase and electron microscopy as applied to tissue culture cells.

Some forty-one authors have contributed, each prominent in his own specialty. The sections are written in considerable detail in most instances. This should enable the reader to carry out the procedures often without reference to the original work. Admittedly however, some recipes are easier to transmit than others and a compendium of this small size cannot always be sufficient unto itself.

HENRY BUNTING

COMPARATIVE PHYSIOLOGY OF THE THYROID AND PARATHYROID GLANDS. By Walter Fleischmann. Springfield, Illinois, Charles C Thomas, 1951. 78 pp. $2.25.

This interesting monograph of seventy-eight pages is Publication Number 118 in the "American Lecture Series" edited by Willard O. Thompson. This series is becoming well known for its pocket-size, up-to-date presentations, bound in flexible black covers.

Fleischmann's monograph brings together many isolated findings in paleo-endocrinology and comparative endocrinology which have not been readily accessible to experimental and practising endocrinologists. The several chapters discuss tunicates and acraniates, fish, amphibia, reptiles, birds, and mammals. Relatively little human data are involved in the last-named section, but a goodly variety of domestic experimental animals is covered.

The presentation is far from purely morphological in that many physiologic findings (natural and experimental) are cited. Radio-active iodine obviously has contributed heavily to this type of material, although there is not much said about the biochemistry of iodine-containing compounds. In general, this work will be of great interest to biologists and workers in the preclinical sciences. As to clinical investigators, it will interest only those who have delved deeply into thyroid and parathyroid problems.

WILLIAM T. SALTER

DISEASES IN OLD AGE. By Robert T. Monroe. Cambridge, Harvard University Press, 1951. xi + 407 pp. $5.00.

With the current increase in life expectancy, geriatrics is coming of age as an important branch of medicine. Dr. Monroe in this monograph gives a thorough statistical analysis of the clinical and pathological findings in almost eight thousand patients over sixty-one years of age. This in itself is a major contribution, and it is made even more valuable by the breakdown of the group according to sex, national background, and profession.

Separate chapters discuss diseases of the various organ systems, which are further considered from the point of view of the patient's body as a whole, possible psychological cause or effect of the condition, and basic therapy.

The author points out the individualistic character of the aged, and this is reëmphasized in the approach to his problems, be it on a medical, psychological, or social level. A good chapter of suggestions rounds out the book, which should become a manual and also a source of reference not only for the geriatrician, but for any medical man who has old patients and hopes to see them grow even older in good mental and physical health.

N. A. H.

FROM A DOCTOR'S HEART. By Eugene F. Snyder. New York, Philosophical Library, 1951. xvii + 251 pp. $3.75.

The publisher states: "This scientifically and spiritually illuminating book answers many practical questions pertaining to the physical and mental health, and touches on the national, racial, and political problems of the day. It is anti-totalitarian; it seeks to promote good inter-faith and inter-racial relationships; it glorifies the general practitioner, the backbone of the medical profession; and the writing is highlighted by a eulogy of America and the ideals of democracy."

Scientifically, it is concerned with coronary heart disease; what the individual can do to prevent it and what the patient can do to make living with it a long-term affair. The medical information and advice given are reliable; the admonition to all doctors, general practitioner and specialist alike, to consider the patient as a whole human being, with a distinct personality, is well taken. Dr. Snyder bemoans the fact that we tend to view our patients as coronaries, cardiacs, appendices, etc., forgetting that we are dealing with and treating entire bodies and people.

Of its attempt to be "spiritually illuminating," the kindest thing this reviewer can say is that it reminds him of a woefully diluted *Peace of Mind* liberally sprinkled with the experiences of a general practitioner. It is an honest, sincere effort and a deeply personal work; as such it deserves our

respect. It is unfortunate that, because of an obvious lack of literary experience and a stylistic inadequacy on the part of the author, it is tiresome reading. For all its sincerity, this book cannot be highly recommended.

J. M. Q.

PARASITIC ANIMALS. By Geoffrey Lapage. Cambridge, Cambridge University Press, 1951. xxi + 351 pp. $4.00.

Parasitic animals is a consideration of parasitism as a way of life for animals. The major portion of the book covers three topics: types of life cycles, effects of this mode of life upon parasites, and effects of parasites upon their hosts. The parasites whose life cycles are discussed all have man or some domestic animal as definitive host. The chapters on the effects of parasitism upon parasites and hosts draw upon the entire animal kingdom for examples.

This book is addressed to the general reader as well as to students and teachers of parasitology. For the general reader the introductory briefing on the zoological classification of parasites is accurate and concise, but in its conciseness may not provide sufficient background for full appreciation of the variety of specific examples in the body of the book. It might be a sufficient stimulus for the acquisition of more background. Teachers will find study of the ecological classification of parasites rewarding in its differences from the usual textbook treatment. The chapters on the effects of parasitism will offer in content little that is novel to these readers, although the manner of organization and presentation provides an admirable pedagogical model. *Parasitic animals* should find its most appreciative audience among students with some zoological education, although even for the layman this authoritative discussion will be readable and stimulating.

J. L. BROOKS

CONNECTIVE TISSUES (Transactions of the First Conference. April 24-25, 1950). Charles Ragan, Ed. New York, Josiah Macy, Jr. Foundation, 1951. 164 pp. $3.25.

This book faithfully records the rather informal meeting organized by the Macy Foundation for the discussion of the problems dealing with the study of connective tissues. The group is limited to twenty-five members and guests but in this instance seventeen scientists, outstanding in their fields of pathology, histochemistry, biochemistry, physiology, etc. participated. The meeting was centered about five papers: Structure and Function of Normal Connective Tissue, by D. Murray Angevine; Pathology of Connective Tissue, Fibrinoid Degeneration, by Granville A. Bennett; Chemistry of Connective Tissue, Polysaccharides, by Karl Meyer; Enzymatically Modified Ovalbumins, by Gertrude E. Perlmann; Effect of ACTH and Cortisone on Connective Tissues, by Charles Ragan. These papers formed the nidus of extensive discussions by the group, which, in addition to the authors, included Frank Fremont-Smith, W. Paul Holbrook, Edward W. Dempsey, Paul György, Molly R. Harrower, Colin M. MacLeod, I. Arthur Mirsky, Albert Szent-Györgyi, Janet Travell, B. W. Zweifach, T. Duckett Jones, and John R. Mote.

Most of the current problems involving connective tissue were touched upon. The discussion of fibrinoid was long and futile. No real difference

could be found between collagen of young and old individuals. Of possible significance was the absence of chondroitin sulfate B in the umbilical cord. The marked individual differences in the action of ACTH and cortisone were pointed out. This book serves to emphasize the meagerness of our knowledge of the connective tissues and the need for a "fresh approach" in their study.

WILLIAM G. BANFIELD

BLOOD CLOTTING AND ALLIED PROBLEMS. (Transactions of the Third Conference, January 23-24, 1950, New York, N. Y.) Joseph E. Flynn, Ed. New York, Josiah Macy, Jr. Foundation, 1950. 224 pp. $3.00.

The prevalent assertion that scientific progress is encumbered by the problem of communication gains special validity when directed to the medical sciences. So profuse is the number of publications appearing that keeping abreast of one's specialty is difficult, reading the literature on other subjects a near impossibility. Yet most authorities would agree with Fremont-Smith that "nature is of one piece" and the "advances in one field are frequently dependent on knowledge derived from quite another." These statements are pertinent to the study of blood clotting.

The integrative efforts of the Josiah Macy, Jr. Foundation are reflected in these transactions: chemists, physiologists, pathologists, internists, and a surgeon contribute to various tangents of the problem of blood clotting. Experimental and therapeutic aspects of some of the newer anticoagulants are described; the use of *zeta*-potential measurements for studying some phases of blood coagulation and several papers on the "so-called prothrombin tests" appear. The informal discussions which followed the presentation of several of these papers at the meeting are included. These discussions emphatically reveal the existing problems, but the very nature of this verbatim reporting makes it necessary for the reader to wade through cumbersome verbiage to find the informative and critical comments.

Like its predecessors this volume should be a necessary acquisition for all medical libraries and required reading for all "clotters." Those only casually interested in blood clotting will gain greater insight into the breadth and complexity of the problem and perhaps become wary of specious conclusions concerning the clotting mechanism.

J .P. G.

PRINCIPLES OF HUMAN GENETICS. By Curt Stern. San Francisco, W. H. Freeman, 1949. xi + 617 pp. 198 figs., 99 tables.

The crying need for a first-rate textbook on human genetics has at last been met by Professor Stern in a volume which combines a thorough exposition of human genetics with a logical presentation of those fundamental principles of genetics which are applicable to all sexually reproducing organisms. It is the outcome of a unique and exciting course which the author began to teach at the University of Rochester and which he has continued at the University of California.

The body of the book opens with a chapter on the biological basis of inheritance which is followed by a treatment of genic action beautifully designed to remove, once and for all, such misconceptions as unit characters, and to indicate that phenotype is the result of complex interactions of genotype and environment. Meiosis is described and analyzed in con-

siderable detail in a chapter which provides the point of departure for many of the succeeding chapters. A brief but sound treatment of probability is followed by chapters on simple single factor inheritance and on lethal and sublethal genes. The practical aspects of this part of the book are then indicated in a chapter on Problems of Genetic Advisors.

Chapter 9 which deals "with some intricate considerations of ratios in small families, ratios in pooled data, and the variability of many phenomena which are influenced by chance" concludes with these words, "we may well be impressed with the ingenuity of the human mind which has been able, by penetrating analysis, to overcome some of the difficulties in understanding caused by the smallness of human families and the working of chance." The reader is impressed with the lucid manner in which the author overcomes the student's difficulties in understanding. One of the most notable features of this book is the able exposition of the statistical aspects of genetics which are fundamental to the study of all populations, human or otherwise. The basic principles of population genetics are introduced by the derivation of the Hardy-Weinberg Law relating to gene frequencies in Chapter 10 and illustrated by its immediate application to the multiple allelic systems underlying human blood group inheritance in Chapter 11. The medico-legal aspects of the blood groups are dealt with succinctly in Chapter 12.

The idea of linkage is introduced by an extensive consideration of sex-linkage in a chapter which gives a rather complete treatment of partially sex-linked genes as well as the better known X- and Y-linked genes. This is followed by the chapter on linkage and crossing over. Chapters 15 and 16 deal with irregular expression of genes and prenatal interactions and should prove of special interest to medical students and practitioners.

Multifactorial inheritance, the basis of many characters, quantitative and otherwise, is given a brief but adequate treatment in Chapter 17 where the examples used are skin color and height in man.

Chapter 1, Consanguinity, continues the more mathematical tenor of Chapters 9, 10, 11, by dealing with systems of mating and their effects on gene frequency in genetic isolates. Chapters 19 and 20 deal with sex determination and the sex ratio, providing a good background from animal genetics in the light of which the situation in man is considered.

The occurrence of mutation and the significance of mutations are dealt with in two chapters remarkable for their approach and for their appraisal of the factors affecting gene and chromosome change. The question of genes and environment, the nature-nurture problem, is dealt with rather extensively using considerable material from the several excellent studies which have been made on identical twins reared apart.

The effects of selection and its bearing on eugenics are accorded a chapter notable for clear presentation of the theoretical effects of selection on gene frequencies under varying situations. The remaining three chapters on the genetic aspects of race, race mixture, and the origin of human diversity form a well-knit unit which summarizes the available information on these subjects and deals with them in line with the development of the principles of population genetics. The final chapter is a kind of *tour de force* in presenting Sewall Wright's conclusions in relatively non-mathematical terms.

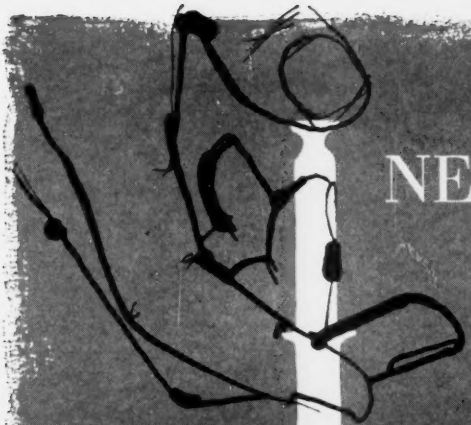
Chapters 9, 10, 11, 15, 18, 21, 24, and 27, which constitute an excellent introduction to modern population genetics, ought to be carefully read by all concerned with problems of human diversity and evolution. The treat-

ment of mutation in Chapters *21-22* is superb, and the same can be said of Chapters *23-27*, which deal with problems of selection, racial diversity, and evolution in the light of population genetics.

Inevitably there must be some omissions, for this is a book on principles rather than materials. A few things are conspicuous by their absence either from the text or index, e.g. red hair, skin color, and tongue curling. Certainly somewhat more in the way of information concerning common differences ought to be included.

The illustrations and tabular data are well chosen and generally well reproduced. There is a fine supply of problems and questions for discussion. The student who has thoroughly mastered this book will be in an excellent position to take up original studies in human genetics. Professor Stern has done such an excellent job and set such a high standard that it will be a long time before this work is surpassed. It is a classic.

D. F. POULSON



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